

**Ascites Tumors—  
Yoshida Sarcoma and  
Ascites Hepatoma (s)**

**MONOGRAPH 16**







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**Ascites Tumors—  
Yoshida Sarcoma and  
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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

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# NATIONAL CANCER INSTITUTE MONOGRAPHS

KENNETH M. ENDICOTT, *Director, National Cancer Institute*

The proceedings of conferences and symposia dealing with cancer or closely related research fields and series of papers on specific subjects of importance to cancer research are presented in these monographs. Send an original, 1 carbon copy, and 2 sets of illustrations (1 mounted) to the Scientific Editor, National Cancer Institute, Bethesda, Maryland 20014. If there is a Conference Editor, manuscripts should be submitted to him.

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**Ascites Tumors—  
Yoshida Sarcoma and Ascites Hepatoma (s)**

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TOMIZO YOSHIDA, *Editor*  
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## FOREWORD

THE first successful transmission of tumor cells to new hosts by means of ascitic fluid was reported by Hesse in 1927. The development of ascites tumors as a useful tool in cancer research and their advantages have been admirably presented in an *Annals of the New York Academy of Sciences* 63: 637-1030, 1956.

Japanese scientists have studied these tumors intensively for more than 20 years. Their interest was aroused by the discovery of the Yoshida sarcoma in 1943, and Dr. Yoshida has summarized their early efforts in the above *Annals* and in the *Journal of the National Cancer Institute* 12: 947-970, 1952. An addendum to this paper by Dr. Nakahara reviews the trends of cancer research in Japan at that time and emphasizes the attention given to ascites tumors.

The aim of this Monograph is to give Japanese investigators the opportunity to present to their English-speaking colleagues, in a single publication, the results of their efforts. Despite the language handicap, they reveal a high degree of effort, enthusiasm, and devotion to science. Their effort, together with the references herein, enables the reader to appreciate the contribution the use of ascites tumors has made to cancer research.

The Board of Editors is privileged to present the Monograph, but its success rests upon the scientific achievements of the authors.

HOWARD B. ANDERVONT, *Scientific Editor*  
*National Cancer Institute*



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## Some Thoughts on Malignant Growths

TOMIZO YOSHIDA, *Sasaki Institute, Sasaki  
Foundation, Tokyo, Japan*

FOR the past 20 years, a group of workers engaged in cancer research in Japan has been endeavoring to further the study of malignant growths. Two papers of Yoshida and his co-workers (12, 14) published in 1943 and 1944 are regarded as the start of the study. The papers dealt with a naive question of a "proper stroma" of malignant tumors. The term "proper stroma" indicated that a new formation of blood vessels or capillary and connective tissues, unlike those pre-existing, accompanied the growth of tumors as the result of infiltration of tumor cells into the surrounding tissues. The attempt to demonstrate histopathologically such proper stroma was not successful. As a result of the study, the investigators became interested in finding the ultimate or essential feature of malignant growth in a pure cell colony of malignant cells without resulting in any tissue formation, *i.e.*, ascites tumors. The so-called Yoshida sarcoma (4) was the first of these tumors produced.

The results of efforts of the group during these years have been reported widely in Japan, but have not been published in such a form as to be readily available to investigators in other parts of the world. Therefore, we are happy to have this opportunity to present our findings, in some detail, to readers of English around the world.

At the end of World War II, only a limited number of experimental tumors were available to those investigators who wished to get cancer research in Japan moving again. One of the materials that played a leading role in the revitalization of cancer research at that time was the so-called Yoshida sarcoma. By 1951, the group succeeded in producing a new type of ascites tumor—the ascites hepatoma (13)—by the ascites conversion of azo dye-induced hepatomas in rats; there are now more than 50 transplantable strains of this ascites tumor. Both Yoshida sarcoma and ascites hepatoma have been distributed widely and are now used by workers in many different laboratories in Japan and other countries.

Today, most cancer research is being carried out with cancer cells as the material, and it may be said that we are heading generally toward an era of the cancer cell. However, no definite criteria yet exist for the

identification of individual cancer cells as such. The only method to make such determinations is the transplantation of the cells into animals. If cell proliferation occurs and kills the animal, it is first determined retrospectively that these cells were cancerous.

Research in laboratories of pathological anatomy and clinical pathology is still centered around the histopathology of cancer tissue with emphasis on a comparison of pathological pictures. Here, the development from normal to malignant growth and the effect on the surrounding healthy tissue are followed. Cancer is considered to be the final step in such development. Pathologists or clinicians who work from this point of view may not always have the same concept, or image, of "cancer" as research workers who study cancer as "individual" malignant cells.

When animal experiments are done to test the chemotherapeutic effect of substances, there is always a question whether ascites tumors or solid tumors should be used. Those who advocate the use of ascites tumors have a cell colony image of cancer, whereas those who prefer solid tumors may have an image of cancer as a tissue.

The final objective of cancer research is human cancer which is almost always in the solid form, and it is easy to understand the emphasis given by investigators to experiments with solid tumors in animals. Somehow, they fear that tests with ascites tumors might not give satisfactory results with reference to human cancers.

There is need to examine the difference between solid growth and ascites growth. This question may best be answered by comparing the ascites tumor with the solid tumor, formed by the subcutaneous transplantation of ascites tumor cells. The latter appears "solid" to the naked eye but is actually a large colony of cancer cells. It does not form tissue comparable to normal tissue. Connective tissues and blood vessels found in the cell colony are not those that developed in support of the cell colony, but those that pre-existed in the normal tissue into which the tumor cells have infiltrated. This is readily observed in the early stages. As tumor cell proliferation progresses, the normal tissue becomes less evident and may finally even be mistaken for stromal portions of tumor. In this "solid tumor," the cells are tightly packed in a small area beneath the skin. Ascites tumor minus ascitic fluid and cells closely packed together would probably look very much like this "solid tumor." Conversely, solid tumors well supplied with intercellular tissue fluid in which individual cells are suspended would look very much like ascites tumors.

Rössle (2) in 1936 and Satomi (3) in 1941 studied solid tumor transplantation, in which the stroma of the graft completely died after transplantation. New growth of transplanted tumor was initiated from a very few tumor cells that survived in the peripheral zone of the graft. Therefore, both techniques of grafting in which different materials are used, such as a piece of tumor tissue and separated individual tumor cells alone, are quite identical in principle.

Thus, there would appear to be no clear difference between ascites growth and solid growth of malignant cells, except for the cell density.

When the amount of tissue fluid is sufficiently great and the number of proliferating cells is adequate, the growth may be called ascites. The growth of ascites tumors is usually accompanied by the formation of numerous solid tumors in the omentum or subperitoneal tissue into which the tumor cells have infiltrated. This phenomenon also occurs in human cancer. Cells may migrate through the body to form "solid" metastatic growth in organs and tissues, or they may enter the peritoneal cavity to produce ascites growth (carcinomatous peritonitis).

In malignant growth, individual cells grow and multiply independently of each other. This is a type of cellular autonomy. It is most clearly reflected in the ascites growth of tumors and definitely demonstrated in the results of the single cell transplantation.

If there is no essential difference between ascites and solid growth, and if the purpose of primary screening in cancer chemotherapy is to determine the effect of chemicals on cancer cells, it would seem that ascites tumors, in which close contact between the chemical and the cell is possible, should be chosen.

There are two general types of cancer materials, *i.e.*, primary tumors that develop within an animal and transplanted tumors. In humans, only primary tumors are involved, and many investigators contend that, if conditions permit, only primary tumors should be used for animal experiments. But, is it really necessary to restrict our materials in this way? When a somatic cell undergoes cancerization within the body of a host the cancer cell thus formed becomes estranged from the surrounding host tissues, as does a cell introduced into the body by transplantation. In both cases, the estranged cells form part of living systems independent of regulation by the host. Investigators would find it difficult to see any difference between primary and transplanted cancer growth.

In the past, it was believed that cancer, or malignant, cells could be defined strictly, for example, that the hepatoma cells of azo dye-induced hepatomas of rats, once established as cancer cells, always had the same nature and the same properties, irrespective of the cells from which they originated. Even though the number of chromosomes in the cancer cells differed, as expected, from the number in normal cells, it was believed that the deviation, whether great or small, would follow a certain, definite rule or direction. Subsequent findings, however, have led us to modify these views.

Since 1951, many transplant strains of ascites hepatomas established from azo dye-induced hepatomas in rats have been successfully maintained. Very early, it was noticed that the shape and size of the island formations, composed of hepatoma cells in the ascitic fluid, and the number of chromosomes varied from strain to strain. In 1954, Satoh (Hiroshi) observed that the responses of ascites hepatoma strains AH 130 and AH 7974 to nitrogen mustard, a chemotherapeutic agent, were dissimilar (5-7). AH 130 was extremely sensitive to the compound and AH 7974 was completely resistant. This finding was quite unexpected, but repeated experiments over a period of 1 year confirmed the results. Up to the

present time, we have established and identified more than 50 transplantable strains of ascites hepatoma, each with different characteristics. The foregoing facts give emphasis to our concept of the individuality of tumors (6, 8).

The basis of cancer research is comparison. First, cancer cells are compared with normal cells. Then, cancer cells are compared with other cancer cells. Ascites hepatomas are suitable materials for such studies and have made it possible to compare, at the cellular level, many different strains of cancers induced in one species under identical conditions.

Of great interest was the finding that there were two separate hepatoma nodules with different characteristics in the same animal (9). Thus, the characteristics of separate hepatoma nodules can be compared in the same animal as well as in different ones. This provides the basis for the consideration that the origin of each cancer can be traced to a separate cancerous cell with individual characteristics (10).

Whether a primary cancer nodule in an organ is of heterogeneous or homogeneous cellular constitution is an interesting problem in cancer pathology. This problem cannot be determined morphologically and the earliest stages of cancer development cannot be detected, regardless of the method employed.

Significant data on successful transplantation rates were obtained in experiments with large numbers of hepatomas (9, 10). With some tumor strains, the rates showed no variation, even after several hundred generations. With other strains, the rates varied at first but later became stabilized at a certain level. Ordinarily, we would expect that the rate of successful transplantation would be low at first, become higher, and then stabilize, and that the survival time would be long at first, become shorter, and then stabilize. In some cases, the patterns for successful transplantation rates and for survival time were quite unexpected.

The foregoing facts suggest that the cellular constitution of the primary tumor to be used for inoculation may be either homogeneous or heterogeneous in nature. If heterogeneous, it is a mosaic of numerous different cell colonies. During their passage through the animal body, competitive or selective elimination occurs, and this is reflected in varying transplantation rates and periods of survival. When, as a result of selection and competition, a certain colony remains, the transplantation rates and the periods of survival are stabilized at a certain level. The rats used in our experiments were not strictly inbred or genetically homogeneous. Therefore, the survival of the cell colony may be influenced by the compatibility of the cell colony with the host animals.

The selection or competition that occurs in different colonies during animal passages of a tumor might also occur during the formation of a tumor in the primary host. Thus, a primary tumor might develop from one of a complicated heterogeneous cell constitution into one of a homogeneous constitution.

In carcinogenesis, numerous cancer cells may originate in one type of tissue, and these cells may differ in nature to a greater or lesser degree.

Each cell could multiply to form a clone. A congregation of clones may constitute the primary tumor formation. The multicentric origin of cancer has been observed histopathologically. This is multicentricity at the cellular level and can be considered as a demonstration of the individuality of a tumor. Thus, an established homogeneous tumor, regardless of size, can be traced to a single cancer cell (10).

Because each ascites hepatoma has distinct characteristics differentiating it from other hepatomas, we are naturally interested in Potter's theory of the "minimal deviated hepatoma" (Morris hepatoma) (1). In our opinion, Morris' hepatoma probably should be described as "lesser deviated" rather than as "minimal deviated." The question also arises whether there might be a "maximal deviated" hepatoma. Although it would be difficult to define maximal and minimal deviation for cancers in general, it might be possible to do this for the more than 50 ascites hepatomas established by us. We should concentrate more effort on studies along this line.

Population analysis of a tumor shows that some clonal sublines are always different or have deviated to some extent from the control population. Furthermore, among many clonal sublines differences are always present. However, a clonal subline that varies from its control population tends to become more and more similar thereto after a period of time. This suggests the existence of a certain range of biological fluctuation in the individual cells constituting an apparently homogeneous tumor cell population.

The dynamic morphology of a separate cancer cell over a period cannot be portrayed as a still picture. The appearance of a cell from one mitosis to the next is manifested as a cycle of movement, whereas the picture from degeneration to necrosis is one of continuous linear movement. Our observations at any given moment are like links in a continually moving chain.

Cancerization, or cancer mutation, of somatic cells is an acquired biological change that has always been considered irreversible. Acquired drug resistance of cancer cells has also been considered as irreversible. This seems to be true in *in vivo* examination. However, it has been demonstrated by Sakurai *et al.*, in *in vitro* experiments, that a strongly acquired resistance can be significantly reversed.

It is believed that a cancer cell is characterized by its rapid multiplication. However, the rate thereof does not seem to be constant and, at times, seems to be drastically slowed down. Metastasis may recur more than 10 years after surgery. Delayed metastasis might be attributed to the condition of the host. It has been observed in some patients that when cancer tissue or lymph node metastasis unavoidably remained after surgical removal of the primary tumor, the tumors later disappeared. In these cases, the tumor-host relationship may be a relevant factor, but whether some changes might have occurred in the cancer cells cannot be easily excluded. Ascites hepatoma AH 62 changed to a slow-growing tumor after storage at  $-80^{\circ}\text{C}$  (8). We are now studying 7 sublines of

Yoshida sarcoma, in which there was a marked slowdown in growth, accompanied by remarkable changes in the ascites characteristics of the tumor (11). These observations are rather rare examples in our long-lasting transplantation work, but this kind of incidence should not be overlooked. Attempts to slow down the rapid multiplicity of cancer cells, to an extreme if possible, may be regarded as a final aim of cancer research.

## REFERENCES

- (1) POTTER, V. R.: Enzymes studied on the deletion hypothesis of carcinogenesis. *In* The Molecular Basis of Neoplasia. Austin, Texas, Univ Texas Press, 1961.
- (2) RÖSSLE, R.: Über die Anfänge der krebsigen Neubildung bei Impfgeschwülsten. Press Akad D Wissenschaft, Phys Math Kl III 1936.
- (3) SATOMI, M.: Über die Struktur des Leberzellenkrebses mit besonderer Berücksichtigung auf das Stroma. (Zur Frage der Stromabildung des transplantierten Hepatoms.) Gann 35: 430-448, 1941.
- (4) YOSHIDA, T.: Studies on an ascites (reticuloendothelial cell?) sarcoma of the rat. J Nat Cancer Inst 12: 947-969, 1952.
- (5) ———: Contributions of the ascites hepatoma to the concept of malignancy of cancer. Ann NY Acad Sci 63: 852-881, 1956.
- (6) ———: Studien über das Ascites-Hepatom. Zugleich ein Beitrag zum Begriff der cellulären Autonomie und der Individualität der einzelnen Geschwulst andererseits. Virchow Arch Path Anat 330: 85-105, 1957.
- (7) ———: Screening with ascites hepatoma. Ann NY Acad Sci 76: 610-618, 1958.
- (8) ———: On the ascites hepatoma. Summary of the results of studies obtained during 10 years from 1951 to 1960. Tokyo J Med Sci 68: 717-748, 1960.
- (9) ———: On the earliest stage of cancer development. A contribution to morphological studies. The Morph. Precurs. Cancer, Div of Cancer Res Univ Perugia, Italy, 1961.
- (10) ———: Zelluläre Multizentrität der Krebsentstehung. Deutsche Med Wschr 88: 2229-2238, 1963.
- (11) YOSHIDA, T., ISAKA, H., and SATOH, H.: Problems of the origin of Yoshida sarcoma. Arzneimittelforschung. In press, 1964.
- (12) YOSHIDA, T., and KIN, T.: Über den histologischen Aufbau des Impfcarcinoms. Gann 37: 343-344, 1943.
- (13) YOSHIDA, T., SATO, H., and ARUJI, T.: Origin of the Yoshida sarcoma. I. Experimental production of "ascites hepatoma" in the rat. Proc Jap Acad 27: 485-492, 1951.
- (14) YOSHIDA, T., SHIMAUCHI, T., TSURUSAKI, H., and SASAKI, J.: Über das Stroma des Sarkoms. Byorigaku Zasshi 3: 122-130, 1944.

## **Transplantability of Yoshida Sarcoma—Result of Serial Transplantation of 1,000 Generations, 1943–1961<sup>1</sup>**

HIROSHI SATOH, *Sasaki Institute, Sasaki Foundation, Tokyo, Japan*

CANCER is said to be a “malignant” tumor and there are various measures of this “malignancy,” but the most characteristic is that once a cancer is produced, cancer cells continue to grow until the host is deprived of its life. This proliferation of cancer cells causes infiltration and destruction of normal tissues, metastases to the whole body, functional disturbance of various internal organs, and worsens systemic conditions to result in cachexia. The degree of these disturbances and changes differs according to the individual, but the result of these phenomena is expressed by the term “malignancy.” Malignancy of cancer should usually be evaluated according to the degree relative to the individual (host) in which this cancer is present. This forms the basis for the “parasitic” aspect of cancer and leads to the understanding that cancer is a pathological phenomenon in which “new cell species” carry on life in an individual.

This malignancy can be defined by two factors: cancer cells and host. It must be considered from two points: changes in the host conditions and change of cancer cells. For example, decrease in the rate of proliferation of cancer cells would lower the malignancy, while changing the nature of the host might strongly affect malignancy. Transplantation is one of the most common methods for observation of proliferation of cancer cells over a long period.

It is over 19 years since the discovery of Yoshida sarcoma, and details of serial transplantation during this period have been recorded and studied. The fact that Yoshida sarcoma can be utilized for observation of transplantation and its course, because of characteristic advantages of ascites tumor, seems to be one of the reasons for its wide use as a new material for experimental tumor studies.

Transplantation of Yoshida sarcoma is made by injection of the tumor ascites into the peritoneum of albino rats, and over 1,000 generations were transplanted since 1943. The present report summarizes the result of transplantation of Yoshida sarcoma during this period.

<sup>1</sup> Partially supported by grant CY-2799 from the National Cancer Institute, National Institutes of Health, Public Health Service.

## YOSHIDA SARCOMA CELLS

The so-called Yoshida sarcoma was discovered in a white rat in June, 1943, in Nagasaki (1). Since then, the tumor ascites has been serially transplanted and the 1,000th generation was reached after 18 years, in June, 1961. Details of this primary source were described previously (2, 3). During these 18 years, the laboratory where these transplant generations were kept has been moved from one place to another, as shown in table 1.

Yoshida sarcoma ascites is turbid, milky, often hemorrhagic, pink or sometimes blood-red. This hemorrhage increases toward the final stage and the hemoglobin value is around 10 to 20 percent. The ascites becomes jaundiced at the end, because bile duct is compressed by infiltration of tumor cells in the liver hilum, which results in systemic jaundice. Volume of the ascites differs according to individual animals, but it is sometimes possible to draw about 10 ml. Usually, a large volume means slightly dilute ascites.

The number of Yoshida sarcoma cells in the ascites is approximately  $10^8$  per ml of the ascites. There is no automotive motion of the cells even under a phase-contrast microscope, and neutrophils are seen moving among these Yoshida sarcoma cells.

When the ascites is to be examined as a smear specimen, the preparation can be made by the technique of blood smears, but since the number of cells in the ascites is numerous, smears must be made thinner so that each of the cells is scattered. (When the ascites is thin and there are few cells, they must be collected by centrifugation or smears made rather thickly and dried rapidly.) The smears are stained by Giemsa or Wright-Giemsa to obtain a clear specimen.

Characteristics of Yoshida sarcoma in such a specimen are as follows: 1) Cells are larger than any others in the blood or peritoneal fluid and have stronger basophilic characteristics in the protoplasm. 2) Nucleus is in an eccentric position and is oval or kidney-shaped and sometimes lobated. 3) Nucleolus is large and clear. Azure granules are present in the cytoplasm, forming the so-called azure rosette.

## TRANSPLANTATION: TECHNIQUE AND NUMBER OF CELLS

Until the 95th generation (April 1946) from discovery, 0.5 to 1.0 ml of the ascites was injected intraperitoneally. From the 96th generation, a glass capillary devised by Haruo Sato was used, and this made transplantation simple and easy. When the glass capillary is pushed into the peritoneum, the ascitic fluid rises into the capillary. (The use of the pipette instead of the injection syringe is recommended even in the collection of a large volume of the ascites. A small amount of heparin will prevent coagulation of the ascites.) Transplantation can be effected by puncture of the peritoneum of a new animal with the ascites blown into

TABLE 1.—Intraperitoneal transplantation of Yoshida sarcoma during 1,000 generations, 1943–1961

Year	Generation	Number of rats	Number of takes*	Number of nontakes*	Remarks
1943	1–19	123	106 (86.2)	17 (13.8)	Discovered at Nagasaki† in June
1944	20–58	376	362 (96.4)	14 (3.6)	
1945	59–88	142	118 (83.1)	24 (16.9)	70th generation, transplanted with a nodule kept in cold room for 7 days
					86th, appeared extinct, then revived, cf animal #1084 (Sendai)
1946	89–120	133	127 (95.5)	6 (4.5)	96th generation, began to use pipette for routine transplantation
1947	121–159	86	81 (94.2)	5 (5.8)	
1948	160–197	60	57 (95.0)	3 (5.0)	171st generation, transplanted with tumor from a rat 1 day after death
1949	198–246	116	109 (93.9)	7 (6.1)	182d, transplanted with 2 cells
					206th, transplanted with tumor kept at 60° C, for 30 minutes
1950	247–299	160	133 (83.1)	27 (16.9)	228th generation, transplanted with 1 cell
					256–262d, passed through mice for 1 month
1951	300–346	245	235 (95.8)	10 (4.2)	394th generation, nitrogen mustard (50 µg/kg)
1952	347–397	190	172 (90.5)	18 (9.5)	426th, transplanted with 1 cell
1953	398–458	327	322 (98.1)	5 (1.9)	511th and 512th, Sarcomycin (100 mg/kg)
1954	459–527	297	297 (100)		593d, mixed with penicillin and streptomycin <i>in vitro</i> (37° C, 30 min)
1955	528–599	293	293 (100)		
1956	600–671	216	216 (100)		
1957	672–740	212	212 (100)		
1958	741–813	152	152 (100)		
1959	814–888	174	173 (99.4)	1 (0.6)	861st generation, passed through a mouse to remove Trypanosoma contamination
1960	889–964	151	151 (100)		
1961‡	965–1000	84	84 (100)		
Total	1–1000	3537	3400 (96.1)	137 (3.9)	

\*Numbers in parentheses indicate percent.

†Places where the tumor was maintained:

1–38th; Dept. of Pathology, Nagasaki Medical College, Nagasaki, Japan;  
 68th–369th; Dept. of Pathology, Tohoku Univ. School of Med., Sendai, Japan;  
 369th–67th and 370th–1,000th; Sasaki Medical Institute, Tokyo, Japan.

‡From January to June, 1961.

the peritoneal cavity. One drop of this ascitic fluid must be made into a smear preparation at the same time.

The volume of transplantation made by this technique can be considered approximately 0.01 to 0.05 ml, which is assumed to contain 1 to 5 million cells.

Cells collected by peritoneal puncture may be calculated by the method of blood cell count with the number of cells to be transplanted thus limited. (The ascites is diluted and needle and syringe are used.)

Gradual decrease in the number of cells to be transplanted to determine how far the number can be decreased is one of the experiments that cannot be made easily unless an ascites tumor is used. Results of transplantation with a few cells were published by Ishibashi (4) in 1950. In the initial period, it was believed that there would be no cancer cells in the circulating blood, but systematic examination often revealed cancer cells in blood smear specimens. However, the number of cancer cells in the blood was relatively small, and this blood transplantation experiment was made in the initial stages of experiments with a few cells. The number of cells transplanted by this means was assumed to be 8,000 to 12,500.

Since cancer is not recognized as a local but as a systemic disease, the presence of cancer cells in the circulating blood was noted with interest in regard to metastasis of cancer, and it became possible to detect a small number of cancer cells by the collection methods. Even in Yoshida sarcoma, presence of cancer cells in the blood was detected by means of this technique 3 days after transplantation. When the transplantation method of the blood was used, cancer cells were detected in the blood 12 to 24 hours after the transplantation (5). Recently a tissue culture technique was applied to detect tumor cells in the circulating blood (6).

Later, it became possible to make this transplantation with a single cell, with the method of calculating the number of transplanting cells by serial dilution of the ascitic fluid, and by transplantation after confirmation of the presence of one single cell under the microscope, with the use of a micromanipulator (4, 7).

All cancer cells are not identical in transplantability, even if the cells are from the same tumor and collected at the same time. Even under one field of a single specimen, there are a variety of cells or cells in various stages of degeneration. From this fact alone, it can be understood that the result of transplantation of one single cell cannot be completely 100 percent. Hosokawa, considering that not all the cells in the glass capillary were blown into the peritoneum, and that some cells still adhered to the glass wall, examined the cells by blowing the ascites sucked into the capillary with a single cell and found this single cell in 175 of 200 tests. The rate of a single-cell transplantation is 30 to 50 percent, including the aforementioned degenerated cells. Cancer produced by the transplantation of a single cell is recorded as a single-cell clone. Most cancer cells in such clones are no different from the parent cells in their properties, but some show a difference in susceptibility to chemical agents (*see* Isaka, this Monograph).

In the 1930's transplantation with a small number of cells was considered unreliable with less than 1 million or reliable with 50,000. However, it is impossible to limit the number of cells without a consideration of the kind and nature of cancer cells and the kind of animals used for transplantation. Transplantation of Yoshida sarcoma has repeatedly succeeded with a single cell. This successful single-cell transplantation is believed to advance analyses on the nature of each single cell.

Results of transplantation with Yoshida sarcoma during the past 18 years are summarized in table 1. Before the 95th generation, when transplantation was carried out with an injection syringe, the number of cells transplanted had not been considered. After the 96th generation, when a glass capillary was used, the number of cancer cells to be transplanted was taken into consideration. In the latter case,  $1$  to  $5 \times 10^6$  cells were transplanted. There was no change in the rate of transplantation before and after the 95th generation. In the initial period, mean survival time was rather long, 13 days. The survival time was similarly 12 to 13 days on the average in the initial period when the glass capillary was used. Such values have not been reproduced later. This result may have some bearing on the composition of tumor cells and on the host-tumor relationship. It is possible that the most malignant of the tumor cells had survived. The longer survival time observed during 1946 and 1947 may have been due to the decrease in the number of tumor cells transplanted, but thereafter the survival period became somewhat shorter, 9 or 10 days. Since 1954, some considerations were given to the animals used for transplantation and animal strains were limited, which resulted in further shortening of the survival period to 7 or 8 days. In recent years, the number of cells to be transplanted has been further decreased to  $10^5$  to  $10^6$  cells, and mean survival time is 8 days. This is believed to be the influence of selective proliferation of cancer cells most suited for growth in connection with host-tumor relationship.

## TISSUE REACTION TO TRANSPLANTATION

When a large amount of Yoshida sarcoma is transplanted into the peritoneal cavity there is a marked proliferation of cells, resulting in a pure culture within a few days after transplantation. Before the culture is pure, intraperitoneal cell reaction (tissue reaction) can be followed periodically by serial observations after transplantation. The predominating cells of the reaction are neutrophils and monocytes. Neutrophils appear about 30 minutes after the transplantation and are active for the next 2 to 3 to 6 hours. This is followed by the appearance of acidophils, whose number reaches the maximum about 24 hours after the transplantation and then decreases gradually. Acidophils are followed by monocytes which reach maximum activity at about 24 hours and end after 72 hours. Yoshida sarcoma cells begin maximum proliferation about 48 hours after the transplantation, after the monocytes appear, and are in pure culture about 72 hours later. Activity of monocytes becomes marked when that

of neutrophils and acidophils begins to decrease. The monocytes show marked hypertrophy and attack leukocytes (macrophages) (tumor cells are not phagocytic). Activity of monocytes occurs in contrast to degenerative disappearance of temporarily increased leukocytes. When the leukocytes disappear, macrophages also disappear.

The reaction disappears rapidly in transplantation with ascites, as shown previously, but, in the subcutaneous transplantation with a piece of solid tumor, reacting cells including neutrophils form a wall surrounding the transplanted tumor. Consequently, most of the tumor mass undergoes necrosis and only the outermost layer continues to grow, infiltrating into the surrounding tissue through the wall of the reacting cells. In the subcutaneous transplantation of ascites tumor, on the other hand, almost perfect transplantation of tumor cells alone is performed and the stroma accompanied by blood vessels is not transplanted. In this case, the "wall" of reacting cells is hardly formed. Thus, cell reaction is not a reaction against the tumor cell itself but must be due to the activity of leukocytes as a foreign body reaction against the stroma or necrotic cells.

Cell reaction in the peritoneum occurs also by intraperitoneal injection of a supernatant of tumor ascites, blood of other rats, or even turpentine oil, which shows that this is exactly the same as a reaction against a foreign substance.

In the later stages of Yoshida sarcoma, intense degeneration and necrosis of tumor cells are noticed, monocytes again increase, and phagocytosis becomes active. These monocytes mostly attack destructive products of tumor cells.

## TRANSPLANTATION RESULTS

After intraperitoneal transplantation of Yoshida sarcoma, most animals die with tumor in about 10 days. Some animals live longer and succumb to tumor, others are cured, *i.e.*, some cured spontaneously are usually classified as negative, but the factors leading to cure are not the same for all animals. In some, the cure is effected by a rapid disappearance of tumor cells; in others, the tumor cells remain a long time before the animal is finally cured. (Only spontaneous cure has been reported in the present case. But it seems important to consider induced cure, in which proliferation of cancer cells may be inhibited by a chemical agent, or host conditions may be so altered as to create a state unfit for cell growth, which finally produces a cure.) Negative growth or spontaneous cure is an abnormal progress of tumor growth and may be taken as an extremely long period elapsing until death. If the animals do not die during the mean survival time, they can be classified as cases of abnormal progress and can be divided into those that finally succumb to tumor death and those that do not die (spontaneous cure). The following chart shows the progress of tumor growth in animals after examination of the so-called negative and positive transplantation of Yoshida sarcoma in rats.

## Chart 1

## I. Normal progress

- A. Inoculated with usual amount of tumor ascites (containing more than 2 million tumor cells). Tumor growth reaches a "nearly pure culture state" within 3 to 4 days, and the hosts die in about 10 days.
- B. Inoculated with 1 to 10<sup>4</sup> tumor cells, which become detectable in approximately 10 days, and the hosts die about 10 days later.

## II. Abnormal progress

- A. Tumor death. (In the group of tumor "take," I and II A are included. The hosts die with abnormally progressing tumor.)
  - 1) Tumor growth reaches a "nearly pure culture state" 3 to 4 days after inoculation, and the hosts survive more than 20 days (twice the average survival time).
  - 2) After reaching a "nearly pure culture state," tumor cells disappear in the ascites once, then reappear after a long interval.
  - 3) After disappearance of tumor cells in the ascites, solid tumor growth is observed in the peritoneal cavity.
  - 4) The hosts die with metastatic tumor growth at distant sites.
- B. Spontaneous regression. (The hosts are cured from tumor and show spontaneous regression.)
  - 5) Metastatic tumors as in (4) regress.
  - 6) Tumor ascites disappears, and solid tumors are observed, then these tumor nodules regress.
  - 7) Tumor growth reaches a "nearly pure culture state," then regresses.
  - 8) No tumor cells grow in the ascites.

Further examination of the results shown in table 1, with consideration of abnormal progress, gave results listed in table 2. Monthly statistics of tumor transplantation, according to the classification shown in chart 1, gives the results shown in table 3.

TABLE 2.—Yearly rate of the transplantation of Yoshida sarcoma inoculated intraperitoneally

Year	Number of animals	Abnormal progress					
		Normal progress		Tumor death		Spontaneous regression	
		Number	Percent	Number	Percent	Number	Percent
1943	123	98	79.7	8	6.5	17	13.8
1944	376	353	94.0	8	2.4	14	3.6
1945	142	112	78.9	6	4.2	24	16.9
1946	133	117	88.0	10	7.5	6	4.5
1947	86	77	89.5	4	4.7	5	5.8
1948	60	56	93.3	1	1.7	3	5.0
1949	116	108	93.1	1	0.8	7	6.1
1950	160	127	79.3	6	3.8	27	16.9
1951	245	223	91.0	12	4.8	10	4.2
1952	190	164	86.3	8	4.2	18	9.5
1953	327	317	98.1	5	0.9	5	0.9
1954	297	297	100				
1955	293	293	100				
1956	216	215	99.5	1	0.5		
1957	212	208	98.0	4	2.0		
1958	152	150	98.7	2	1.3		
1959	174	171	98.2	2	1.2	1	0.6
1960	151	150	99.3	1	0.7		
1961	84	84	100				
Total	3537	3320	94.3	80	1.8	137	3.9

TABLE 3.—Monthly result of intraperitoneal transplantation of Yoshida sarcoma

Year	Month	Number of animals	Normal progress		Tumor death		Spontaneous regression	
			Number	Percent	Number	Percent	Number	Percent
1943 (Nagasaki)	6	8	4	50.0	2	25.0	2	25.0
	7	9	5	55.6			4	44.4
	8	22	16	72.8	3	13.6	3	13.6
	9	20	18	90.0			2	10.0
	10	29	22	75.9	2	6.9	5	17.2
	11	13	13	100.0				
	12	22	20	91.0	1	4.5	1	4.5
1944	1	28	27	96.4	1	3.6		
	2	25	25	100.0				
	3	38	32	84.2	6	15.8		
	4	24	24	100.0				
	5	44	44	100.0				
	6	28	27	96.4			1	3.6
	7	45	48	84.4			7	15.6
(Tokyo)	8	19	15	78.9			4	21.1
	9	17	15	88.2			2	11.8
	10	4	3	75.0	1	25.0		
	11	52	51	98.1	1	1.9		
	12	52	52	100.0				
1945	1	44	42	95.6	1	2.3	1	2.3
	2	18	16	88.9			2	11.1
	3	8	6	75.0	1	12.5	1	12.5
	4	12	9	75.0			3	25.0
	5	16	15	93.7			1	6.3
	6	8	4	50.0			4	50.0
	7	9	8	88.9			1	11.1
(Sendai)	8	11	7	63.6	1	9.1	3	27.3
	9	5	2	40.0				
	10	2			1	50.0	1	50.0
	11							
	12	9	3	33.4	2	22.2	4	44.4

1946	1	19	18	94.7	2	22.2	1	5.3
	2	9	6	66.7	1	11.1	1	11.1
	3	13	12	88.9				
	4	13	10	92.3	1	7.7	1	7.7
	5	13	12	76.9	1	7.7	2	15.4
	6	13	12	92.3	3	18.7	1	6.3
	7	16	12	75.0				
	8	13	13	100.0	1	6.7		
	9	15	14	93.3				
	10	4	4	100.0	1	25.0		
	11	4	3	75.0				
	12	5	5	100.0				
1947	1	6	3	50.0	1	16.7	2	33.3
	2	4	4	100.0				
	3	5	5	100.0				
	4	6	6	100.0				
	5	6	6	100.0				
	6	9	8	88.9	1	11.1		
	7	2	1	50.0			1	50.0
	8	12	11	91.7			1	8.3
	9	8	8	100.0				
	10	13	13	100.0	1	11.1		
	11	9	8	88.9	1	16.7	1	16.7
	12	6	4	66.6				
1948	1	10	8	80.0			2	20.0
	2	1	1	100.0				
	3	8	8	100.0				
	4	5	5	100.0				
	5	5	5	100.0				
	6	6	5	83.3				
	7	5	4	80.0	1	20.0	1	16.7
	8	3	3	100.0				
	9	2	2	100.0				
	10	5	5	100.0				
	11	4	4	100.0				
	12	6	6	100.0				

TABLE 3.—Monthly result of intraperitoneal transplantation of Yoshida sarcoma—Continued

Year	Month	Number of animals	Normal progress		Tumor death		Spontaneous regression	
			Number	Percent	Number	Percent	Number	Percent
1949	1	6	6	100.0				
	2	6	4	66.7			2	33.3
	3	6	2	33.3			4	66.7
	4	12	12	100.0				
	5	12	10	83.4	1	8.3	1	8.3
	6	10	10	100.0				
	7	11	11	100.0				
	8	19	19	100.0				
	9	11	11	100.0				
	10	9	9	100.0				
	11	5	5	100.0				
	12	9	9	100.0				
1950	1	8	6	75.0			2	25.0
	2	8	7	87.5	1	12.5		
	3	12	8	66.7			4	33.3
	4	16	8	50.0	2	12.5	6	37.5
	5	18	14	77.8			4	22.2
	6	8	8	100.0				
	7	16	13	81.2	1	6.3	2	12.5
	8	10	10	100.0				
	9	12	10	83.4	1	8.3	1	8.3
	10	16	14	87.5			2	12.5
	11	19	16	84.2	1	5.3	2	10.5
	12	17	13	76.5			4	23.5
1951	1	13	13	100.0				
	2	10	10	100.0				
	3	14	11	78.7	2	14.2	1	7.1
	4	11	11	100.0				
	5	17	14	82.4			3	17.6
	6	19	17	89.5			2	10.5
	7	42	38	90.4	2	4.8	2	4.8
	8	27	22	81.5	5	18.5		
	9	29	29	100.0				
	10	24	20	83.3	3	12.5	1	4.2

	11	20	19	19	95.0	1	5.0
	12	19	19		100.0		
1952	1	14	12		85.7	2	14.3
	2	22	17		77.3	2	9.1
	3	21	18		85.6	1	4.8
	4	13	11		84.6	2	15.4
	5	23	15		65.2	6	26.1
	6	19	16		84.2	3	15.8
(Tokyo)	7	11	11		100.0		
	8	12	12		100.0		
	9	12	12		100.0		
	10	9	9		100.0		
	11	12	12		100.0		
	12	22	19		86.4	2	9.1
1953	1	55	49		89.1	1	1.8
	2	21	21		100.0	5	9.1
	3	17	17		100.0		
	4	24	22		91.7	2	8.3
	5	35	34		97.1	1	2.9
	6	30	29		96.7	1	3.3
	7	31	31		100.0		
	8	15	15		100.0		
	9	36	36		100.0		
	10	22	22		100.0		
	11	20	20		100.0		
	12	21	21		100.0		
1954	1	14	14		100.0		
	2	20	20		100.0		
	3	14	14		100.0		
	4	18	18		100.0		
	5	21	21		100.0		
	6	21	21		100.0		
	7	16	16		100.0		
	8	14	14		100.0		
	9	36	36		100.0		
	10	39	39		100.0		
	11	40	40		100.0		
	12	44	44		100.0		

TABLE 3.—Monthly result of intraperitoneal transplantation of Yoshida sarcoma—Continued

Year	Month	Number of animals	Normal progress		Tumor death		Spontaneous regression	
			Number	Percent	Number	Percent	Number	Percent
1955	1	36	36	100.0				
	2	29	29	100.0				
	3	21	21	100.0				
	4	19	19	100.0				
	5	21	21	100.0				
	6	13	13	100.0				
	7	26	26	100.0				
	8	33	33	100.0				
	9	29	29	100.0				
	10	21	21	100.0				
	11	30	30	100.0				
	12	15	15	100.0				
1956	1	24	24	100.0				
	2	13	13	100.0				
	3	12	12	100.0				
	4	10	9	90.0	1	10.0		
	5	15	15	100.0				
	6	18	18	100.0				
	7	20	20	100.0				
	8	27	27	100.0				
	9	30	30	100.0				
	10	12	12	100.0				
	11	16	16	100.0				
	12	19	19	100.0				
1957	1	18	18	100.0				
	2	15	15	100.0				
	3	18	17	94.0	1	6.0		
	4	16	16	100.0				
	5	18	18	100.0				
	6	17	17	100.0				
	7	15	15	100.0				
	8	18	15	83.3	3	16.7		
	9	20	20	100.0				
	10	21	21	100.0				
	11	18	18	100.0				
	12	18	18	100.0				

1958	1	15	100.0			
	2	18	100.0			
	3	12	100.0			
	4	12	100.0			
	5	12	100.0			
	6	10	100.0			
	7	12	100.0			
	8	14	85.6	2	14.4	
	9	11	100.0			
	10	14	100.0			
	11	11	100.0			
	12	13	100.0			
1959	1	12	100.0			
	2	12	91.6	1	8.4	
	3	13	100.0			
	4	17	100.0			
	5	12	100.0			
	6	12	100.0			
	7	12	100.0			
	8	11	91.6	1	8.4	
	9	18	100.0			
	10	29	100.0			
	11	13	100.0			
	12	14	100.0			
	12	12	91.6	1	8.4	
1960	1	12	100.0			
	2	12	100.0			
	3	12	91.6	1	8.4	
	4	12	100.0			
	5	12	100.0			
	6	12	100.0			
	7	14	100.0			
	8	12	100.0			
	9	12	100.0			
	10	12	100.0			
	11	14	100.0			
	12	16	100.0			
1961	1	14	100.0			
	2	14	100.0			
	3	14	100.0			
	4	13	100.0			
	5	14	100.0			
	6	15	100.0			
Total		3537	94.3	80	1.8	137
						3.9

## ANIMALS FOR TRANSPLANTATION

## Sex

Correlation between the sex of the animals used and the transplantability was examined in the animals transplanted during 1950 to March, 1953, and the results are summarized in table 4. There was no significant difference in transplantation rate between both sexes. Transplantation rate was almost 100 percent for all animals used since that time and there was no difference between the two sexes.

TABLE 4.—Transplantability of Yoshida sarcoma in male and female rats

Year	Number of rats	Sex	Abnormal progress						
			Normal progress			Tumor death		Spontaneous regression	
			Number	Per-cent		Number	Per-cent	Number	Per-cent
1950	160	Male	102	83	81.3	3	3.0	16	15.7
		Female	58	44	75.8	3	5.2	11	19.0
1951	245	Male	130	119	91.5	6	4.6	5	3.9
		Female	115	104	90.5	6	5.2	5	4.3
1952	190	Male	107	93	86.9	3	2.8	11	10.3
		Female	83	71	85.5	5	6.0	7	8.5
1953*	93	Male	41	40	97.5	1	2.5	0	---
		Female	52	47	90.4	0	---	5	9.6
Total	688	Male	380	335	88.2	13	3.4	32	8.4
		Female	308	266	86.4	14	4.5	28	9.1

\*From January to March.

## Weight and Age

Weight and age were related to the rate of transplantation in animals used from 1950 to August, 1955. (Actually, observations should have been made according to the number of days after birth, but this was not clear in animals procured from a dealer at that time. Most of the animals weighed around 60 g after 2 months and 300 to 350 g after 12 months, with males weighing 20 to 30 g more than females. Thus the approximate age of an animal can be determined from its body weight.) Results are shown in table 5. The animals were classified according to their weight at the time of transplantation (figures below 10 g were discarded). It has generally been said that transplantation rate was higher in younger animals and the rate gradually became lower as the animals grew older. From the results obtained (table 5), however, such a simple conclusion could not be drawn as far as the transplantation of Yoshida sarcoma in a usual dose was concerned.

TABLE 5.—Body weight of animals and transplantability of Yoshida sarcoma

Body weight (g)	Number of rats	Abnormal progress					
		Normal progress		Tumor death		Spontaneous regression	
		Number	Percent	Number	Percent	Number	Percent
10-150	1132	1051	93.1	29	2.3	52	4.6
160	33	33	100.0				
170	22	20	91.0	1	4.5	1	4.5
180	18	17	94.5			1	5.5
190	12	12	100.0				
200	15	15	100.0				
210	16	15	93.7			1	6.3
220	7	6	85.8			1	14.2
230	7	7	100.0				
240	5	5	100.0				
250	4	3	75.0			1	25.0
260	5	4	80.0	1	20.0		
270	2	2	66.6			1	33.4
280	1	1	100.0				
290	4	4	100.0				
300	3	3	100.0				
310	3	3	100.0				
320	3	3	100.0				
340	2	2	100.0				
350	1	1	100.0				
380	2	2	100.0				
390	1	1	100.0				
400	2	1	50.0			1	50.0
420	2	2	100.0				
430	1					1	100.0
510	1	1	100.0				
160-510	176	166	94.3	2	1.1	8	4.5
Total	1308	1217	93.0	31	2.5	60	4.5

### Individual Susceptibility

Tumor production and transplantation have been studied by many workers with genetically pure, inbred, *i.e.*, pure-strain animals, and it is known that the rate of transplantation is generally high and constant in these animals. Yoshida sarcoma was found in a noninbred rat, and commercially available rats were used for transplantation until 1953. Even with such animals, the average rate of transplantation was quite high. Transplantation experiments were usually carried out with animals procured at random so that the transplantation rate was extremely good at some period, while the rate became very poor with animals procured at a different period or from a different dealer. This might point out the importance of individual difference in animals and suggests the role of genetical factors, considering the use of pure-strain animals.

Transplantation rate was therefore examined in animals obtained by inbreeding of offspring from naturally cured parents (table 6).

Some  $F_1$  animals are cured spontaneously at a high rate (expts. 1-3), and some show almost normal progress (expts. 19-26), resulting in tumor

TABLE 6.—Transplantability of Yoshida sarcoma in inbred offspring from rats with spontaneous regression of tumor

F <sub>1</sub>				F <sub>2</sub>				F <sub>3</sub>			
Parent No.			I*	II-A	II-B	Parent No.			I	II-A	II-B
	Male	Female					Male	Female			
1	†1750	1749		†6(100)		27 28 29	†1750 X195 X379	X381 X196 X381	7(87) 8(100) 1(13)	44	6(86) 1(14)
2	1747	1729		6(100)							
3	1750	1691		4(31) 9(69)							
4	1750	X231		2(100)							
5	5121	1725	1(10)	6(60) 3(30)							
6	K13	1691	1(10)	6(60) 3(30)		30 31 32	X113 X113 X116	X109 X121 X121	5(56) 3(19) 2(15)	4(44) 5(31) 1(8)	6(100)

## ASCITES TUMORS

8	4064 1750	4047 6523	1(17) 2(27)	4(66) 4(45)	1(17) 3(33)	36	1750	X324	7(100)			
9	1750	5180	4(27)	3(20)	8(53)	37 38	1750 1750	X270 X272	1(33) 2(33)	1(34) 4(67)	1(33)	
10	1750	5179	5(36)	1(7)	8(57)	39 40 41 42	1750 1750 1750 1750	X277 X276 X280 X279	5(100) 3(100) 1(25) 2(100)		49 50 51	X481 X481 1750
11	1750											X483 X276 X439
12	1747	6502	5(45)	2(18)	3(37)							
13	1750	6503	3(50)	3(50)								
14	K13	1690	4(57)	3(43)								
15	K13		3(60)		2(40)							
16	1750	5173	5(72)	1(14)	1(14)							
17	5121	1714	8(73)	2(18)	1(9)							
18	5121	1720	10(77)	3(23)		43	1750	X294				
19	5145	5114	7(88)		1(12)							
20	K13	6011	8(100)									
21		6516	12(100)									
22	1747	6031	9(100)									
23	1750	X237	4(100)									
24	5145	1725	7(100)									
25	X150	X160	11(100)									
26	5145	5125	7(100)									

•I. Tumor death with normal progress; II-A. tumor death with abnormal progress; II-B. spontaneous regression with abnormal progress (see chart 1).

<sup>†</sup>These are experiment numbers.

Percentage of animals in parentheses after number of animals.

death. It should be noted here, however, that the survival time has been lengthened abnormally in many animals dying with tumor. There are numerous  $F_1$ ,  $F_2$ , and  $F_3$  animals which succumb to tumor after a long period (abnormal progress).

In experiment 1, all 6 animals of  $F_1$  were cured spontaneously, while in  $F_2$ , the litters from 3 pairs of parents show different progress: Those of the first group (expt. 27) were cured spontaneously, those of the second group (expt. 28) took the abnormal course of tumor death, and 87 percent of the third group (expt. 29) took the normal progress of tumor death.

Although observation of individuals does not necessarily give the same result, summarized results of all the experiments (table 7) indicate that, in  $F_1$ , positive transplantation rate, taking the normal course, was lowered to 53.3 percent, which is extremely low for transplantation of Yoshida sarcoma. This rate was further lowered to 45.6 percent in  $F_2$  and increased to 53.3 percent in  $F_3$ .

TABLE 7.—Correlation between transplantability of Yoshida sarcoma and sex in inbred offspring from parents with spontaneous regression of tumor

Generation	Sex	Number of animals	Abnormal progress					
			Normal progress		Tumor death		Spontaneous regression	
			Num- ber	Per- cent	Num- ber	Per- cent	Num- ber	Per- cent
$F_1$	Male	115	60	52. 2	26	22. 6	29	25. 2
	Female	114	62	54. 4	22	19. 3	30	26. 3
	Total	229	122	53. 3	48	20. 9	59	25. 8
$F_2$	Male	73	31	42. 5	19	26. 0	23	31. 5
	Female	76	37	48. 7	16	21. 0	23	30. 3
	Total	149	68	45. 6	35	23. 5	46	30. 9
$F_3$	Male	25	12	48. 0	3	12. 0	10	40. 0
	Female	20	12	60. 0	4	20. 0	4	20. 0
	Total	45	24	53. 3	7	15. 6	14	31. 1
Total males		213	103	48. 3	48	22. 5	62	29. 2
Total females		210	111	52. 9	42	20. 0	57	27. 1
Grand total		423	214	50. 6	90	21. 3	119	28. 1

Differences of transplantation rate according to sex and its relation to body weight were examined with all  $F_1$ ,  $F_2$ , and  $F_3$  animals. There seemed to be no significant difference according to the sex (table 8), but a slight and rather interesting effect was seen in the body weight of the animals. Comparatively young animals were used for this experiment, and the rate of transplantation was definitely and markedly lower than that in the controls (*cf* table 5). This seems to suggest that the resistance or susceptibility of an animal is affected by some genetic factors such as the breeding strain.

Kaziwara made the same examination with  $F_1$ ,  $F_2$ ,  $F_3$ , and  $F_4$  litters starting from a pair of the same parent, and his results are given in table 9

TABLE 8.—Transplantability of Yoshida sarcoma and body weight of inbred offspring from resistant parents

Body weight (grams)	Number of animals	Total									
		F <sub>1</sub>				F <sub>2</sub>				F <sub>3</sub>	
		I*	II-A	II-B	I	I	II-A	II-B	I	II-A	II-B
10	1	1									
20	13	3	2	2	6				1	100.0	
30	85	26	6	20	21	7			9	69.2	
40	94	15	11	13	28	11		5	47	55.3	
50	53	13	6	13	3	8		16	43	45.7	
60	45	20	3	3	4	2			18	34.0	
70	50	17	8	3	3	6		6	27	60.0	
80	40	15	7	2	3	1		4	28	56.0	
90	29	9	5	3	3			2	24	60.0	
100	7	3		1				1	14	48.3	
110	2	1							1	50.0	
120	3			1							
130	2			1							
Total	423	122	48	59	68	35	46	24	7	14	214
										50.6	90
										21.3	119
											28.1

\*I: normal progress; II-A: tumor death with abnormal progress; II-B: spontaneous regression with abnormal progress.

(8). This table shows that abnormal progress does not increase with passage of generations. Furthermore, the rate of abnormal progress in the offspring of the parents cured spontaneously does not increase compared to that in the progeny of the parents, male or female of which was cured spontaneously (table 10).

TABLE 9.—Transplantability in inbred offspring from a pair of resistant parents (reported by Kaziwara)

Generation	Number of animals	Abnormal progress					
		Normal progress		Tumor death		Spontaneous regression	
		Num-ber	Per-cent	Num-ber	Per-cent	Num-ber	Per-cent
F <sub>1</sub>	23	10	43.5	3	13.0	10	43.5
F <sub>2</sub>	39	23	59.0	6	15.4	10	25.6
F <sub>3</sub>	7	3	43.0	1	14.0	3	43.0
F <sub>4</sub>	34	16	47.0	6	17.6	12	35.4
Total	103	52	50.0	16	15.5	35	33.9

TABLE 10.—Transplantability in the young

Parents			Animals					
			I*		II-A		II-B	
			Num-ber	Per-cent	Num-ber	Per-cent	Num-ber	Per-cent
Male	Female	Num-ber of young						
I*	II-B	6	2	33. 3	3	50. 0	1	16. 6
II-A	II-B	13	5	38. 4	1	7. 7	7	53. 9
II-B	I	11	6	54. 7	1	9. 0	4	36. 3
II-B	II-B	39	23	59. 0	6	15. 4	10	25. 6

\*I: tumor death with normal progress; II-A: tumor death with abnormal progress; II-B: spontaneous regression with abnormal progress (see chart 1).

It may be concluded from the foregoing experiments that there is no evidence for gradual increase of resistance by inbreeding, but that the offspring from parents with a tendency for spontaneous cure tend to become more resistant to tumor growth. Even if the offspring die with tumor, survival time until tumor death increases abnormally.

Addendum

1) *Wistar rats*.—Albino rats of the Wistar strain are used a great deal in Japan as the pure-strain albino rats. There are many sublines according to breeders and their transplantation rate differs markedly, as shown in table 11. Therefore, there is a difference in transplantability of Yoshida sarcoma even among rats of the Wistar strain.

2) *Various strains of rats*.—Haruo Sato examined transplantability of Yoshida sarcoma in various strains of rats during his stay at the National Cancer Institute, Bethesda, Maryland, USA (9). Results of his observations are shown in table 12.

3) *Donryu rats*.—Periodic statistics of serial transplantation of Yoshida sarcoma showed that the results were quite good and constant after 1954. This is because the experiments were carried out with the so-called Donryu rats, a group bred from a pair of white rats procured from a breeder in Ohta City, Gumma Prefecture, by Ryuichi Sato of that city in 1950. Transplantation rate of Yoshida sarcoma reported by several researchers using Donryu rats is listed in table 13.

TABLE 11.—Intraperitoneal transplantation of Yoshida sarcoma in Wistar rats

Group	Source	Number of animals	Abnormal progress					
			Normal progress		Tumor death		Spontaneous regression	
			Num-ber	Percent	Num-ber	Percent	Num-ber	Percent
1	Hokkaido University	38	15	39.5			23	60.5
2	Tokyo (1)	40	30	75.0	6	15.0	4	10.0
3	Tokyo (2)	20	15	75.0	5	25.0		

TABLE 12.—Intraperitoneal transplantation of Yoshida sarcoma in various strains of rats (reported by Haruo Sato)

Strain	Initial proliferation of tumor cells*	Number of animals	Take		No take	
			Num-ber	Percent	Num-ber	Percent
Japanese	+++	125	118	98	7	6
M 520	+++	295	253	86	42	14
M 520 × AC	+++	213	174	82	39	18
Sprague-Dawley	+++	104	54	52	50	48
Holtzman	+++	87	44	51	43	49
Buffalo	+++	51	8	16	43	84
Wistar	++	48	4	8	44	92
Osborne-Mendel	+++	73	6	8	67	92
NIH Black	+++	25	2	8	23	92
Fischer	+	35	0	0	35	100
O'Grady	+++	45	0	0	45	100
Albany	+	38	0	0	38	100

\*+++ Proliferation of tumor cells reached a state of nearly pure culture. ++ Tumor cells proliferated slightly. + Tumor cells were alive for a few days in the ascites but did not proliferate.

TABLE 13.—Transplantability of Yoshida sarcoma in Donryu rats

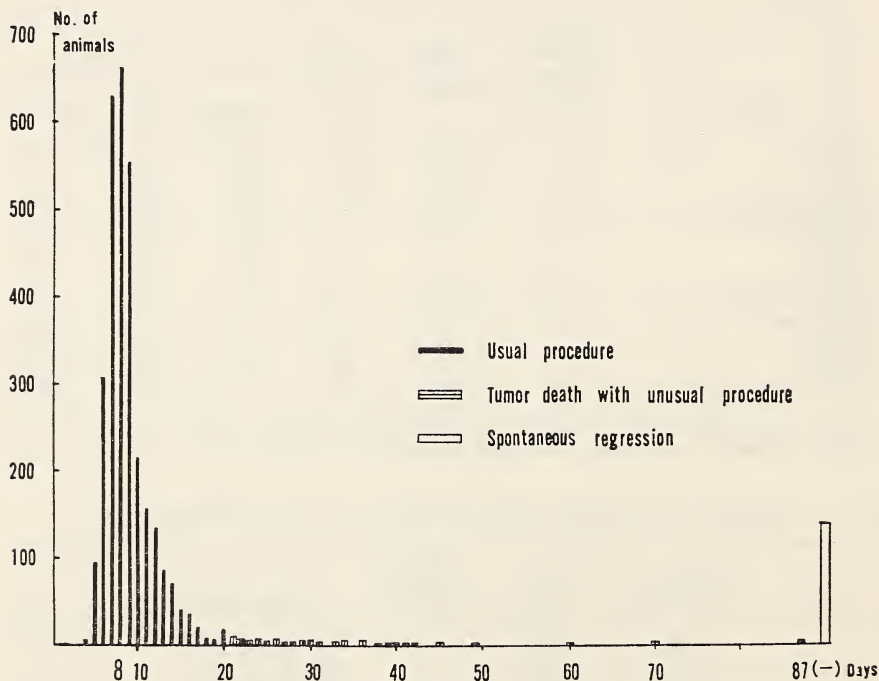
Number of animals	Takes (%)	Average survival (days)	Reported by:
927	99.9	8	Ryuichi Sato
1036	100.0	7	Hiroshi Satoh
122	100.0	8	Hidehiko Isaka
164	99.4	7	Hidehiko Isaka
150	100.0	8	Haruo Sato
686	99.1	8	Kyo Kaziwara

## SURVIVAL PERIOD

The yearly survival period of animals transplanted with Yoshida sarcoma intraperitoneally is illustrated in text-figure 1, and the results in 1,000 serial transplant generations from 1943 to 1961 are shown in text-figure 2. The percentage survival rate is given in text-figure 3. It can be seen from these text-figures that the result of transplantation has become quite uniform since 1954 when Donryu rats were used. This is very important as a control experiment with Yoshida sarcoma. As a general principle, transplantation of a tumor produced in a pure-strain animal into the same pure-strain animal gives a very high rate of transplantation, sometimes recording 100 percent takes. However, there is still quite a wide range of survival period. The combination of Yoshida sarcoma and Donryu rat is a valuable one.

Survival period of animals inbred from spontaneously cured parents is shown in text-figure 4, and its percentage survival rate is given in text-figure 5. These text-figures show clearly the increase in resistance of these animals to transplantation.

In general, the mean life of a tumor animal is expressed simply as the arithmetic mean of a number of days until tumor death of the positive animals. When the number with abnormal progress is quite small, this



TEXT-FIGURE 1.—Survival of rats after intraperitoneal transplantation of Yoshida sarcoma. Days indicate mode of survival period; (—) indicates spontaneous regression with abnormal progress.

arithmetic mean agrees approximately with the maximum frequency (mode) of the survival period. It also agrees with the date on which the percentage survival goes below 50 percent (the day 50 percent of the experimental animals die) and also with the median value of survival period (table 14). When there is a large number with abnormal progress, there is a marked difference among the foregoing four values. Even if 1 animal of the group lives for 100, 150, or longer days before tumor death, this value will give marked increase in the arithmetic mean (table 15).

The percentage survival curve gives some information on the range and width of the survival period of animals with tumors. When therapeutic experiments are carried out with various chemicals, the percentage

TABLE 14.—Difference in average survival time, calculated in various ways, of rats with Yoshida sarcoma

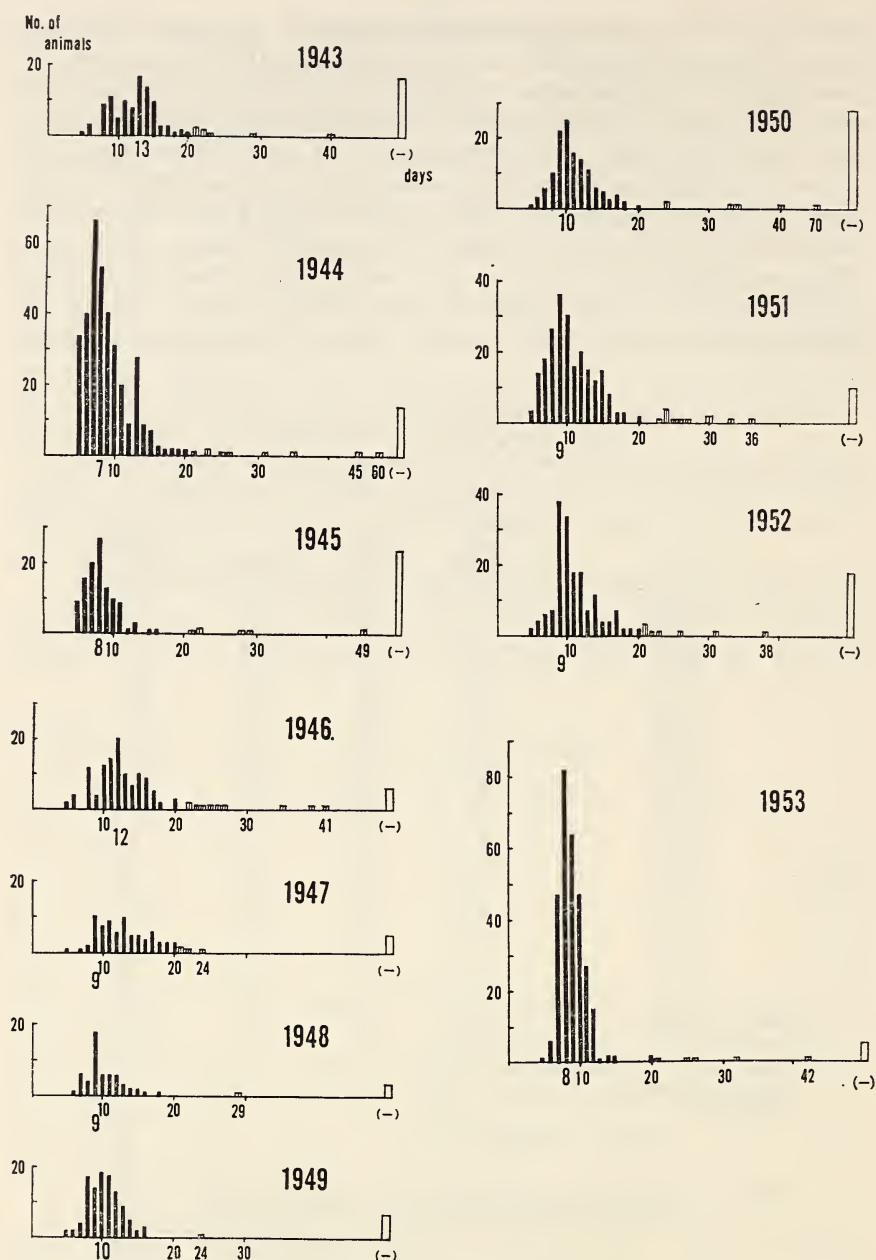
Year	Survival time (days)			
	Mode of dead animals	50% survival	Median	Arith-metical mean
1943	13	13	13	13
1944	7	8	8	9
1945	8	8	9	9
1946	12	12	12	13
1947	9	13	13	13
1948	9	9	9	12
1949	10	10	10	11
1950	10	11	12	12
1951	9	10	10	11
1952	9	10	11	12
1953	9	9	8	10
1954	7	7	7	7
1955	7	7	7	8
1956	7	8	8	8
1957	7	7	7	8
1958	8	8	8	8
1959	8	7	7	7
1960	7	7	7	8
1961	8	8	8	8
Average 1943-53*	9	10	9	11
Average 1954-61†	7	7	7	8

\*Noninbred Japanese rats.

†Donryu rats.

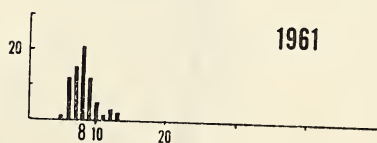
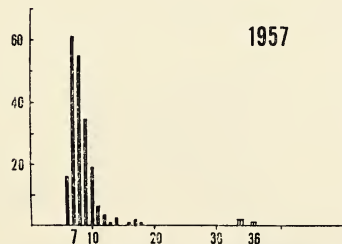
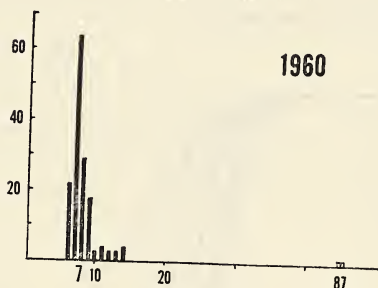
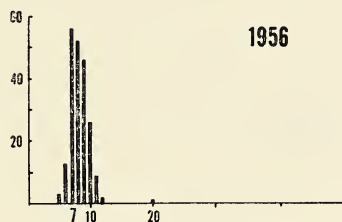
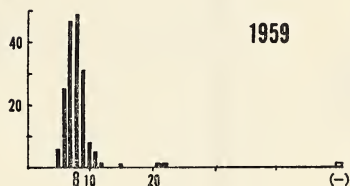
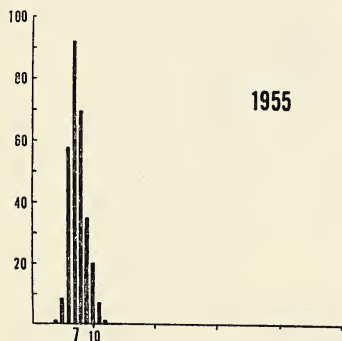
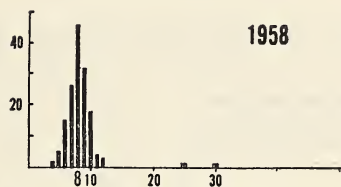
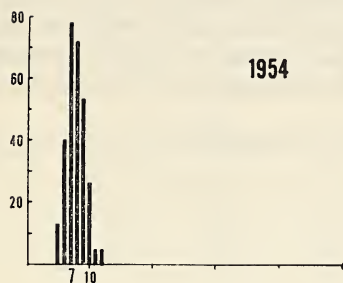
TABLE 15.—Difference in average survival time, calculated in various ways, of inbred offspring from resistant parents

Generation	Survival time (days)			
	Mode of dead animals	50% survival	Median	Arithmetic mean
F <sub>1</sub>	12	17	13	24
F <sub>2</sub>	13	22	14	20
F <sub>3</sub>	12	16	11	14
Total	12	18	14	22



TEXT-FIGURE 2.—Survival period of rats after intraperitoneal transplantation

survival curve can easily be compared with the variation in survival period. Mean life, expressed by median value or mode, seems to be much better than the arithmetic mean as direct expression of the nature of this tumor.



of Yoshida sarcoma during 1,000 generations, 1943-1961.

As has been stated previously, mean life of tumor animals extended to 12 or 13 days at the time of the discovery of Yoshida sarcoma, or when the glass capillary technique was first used. This is possibly related to the constitution of the animals used and number of cells transplanted.

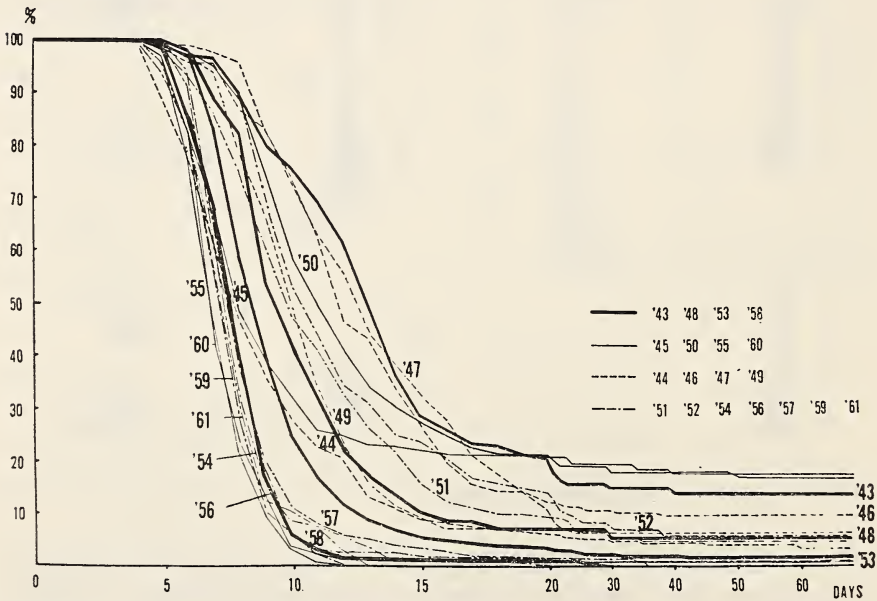
#### ASCITES TUMORS

Yoshida sarcoma was transplanted in Donryu rats with a decrease in the number of tumor cells inoculated, to a minimum of 100 cells, and the results are given in table 16. This shows that the survival period becomes longer as the number of transplanted cells decreases, and also that the

TABLE 16.—Transplantation of various numbers of Yoshida sarcoma cells in Donryu rats

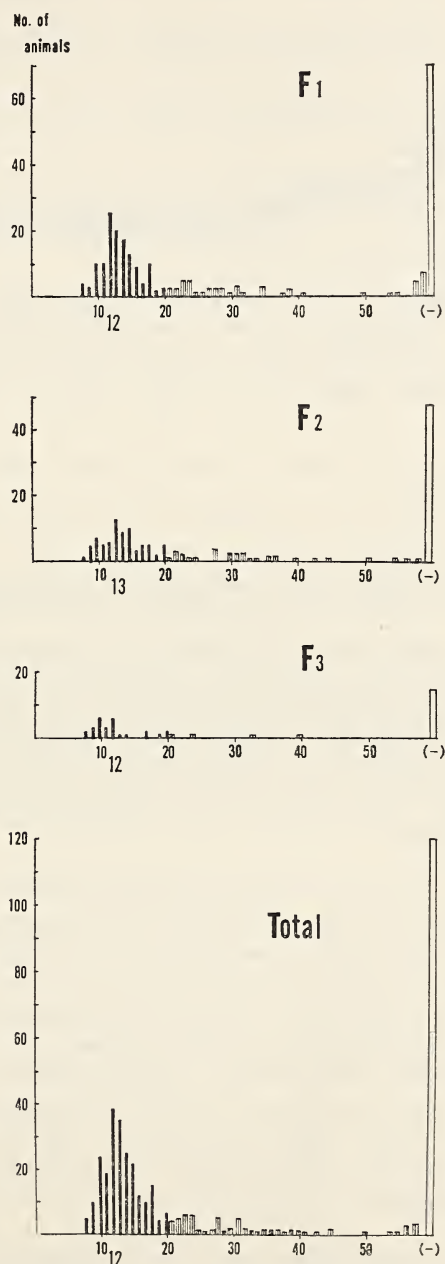
Number of cells transplanted		10 <sup>7</sup>	10 <sup>6</sup>	10 <sup>5</sup>	10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>
Number of animals		10	10	10	10	10	10
Survival time after inoculation (days)	6	4*					
	7	4	2				
	8	2	4				
	9		4				
	10			6	1		
	11			4	3		
	12				3		
	13				2		1
	14				1		
	15					1	2
	16					3	2
	17					3	2
	18					1	
	19						2
	20						
	21					1	
Number of animals that died with tumor		10	10	10	10	10	9
Transplantation rate		100	100	100	100	100	90

\*Number of rats that died.

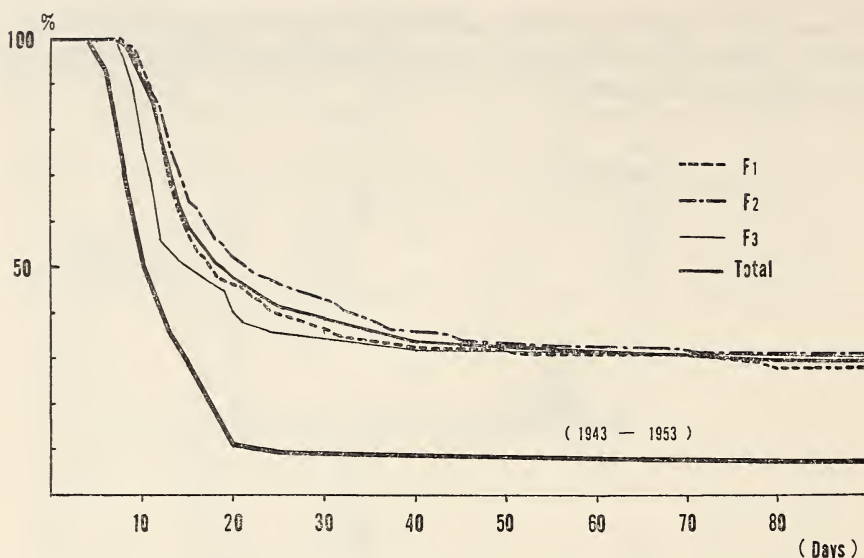


TEXT-FIGURE 3.—Survival percentage of rats after intraperitoneal transplantation with Yoshida sarcoma. Each curve indicates the result for each year, 1943–1961.

decrease in the number of cells tends to give wider range in the survival period. These data offer important information regarding the effect of chemotherapeutic agents.



TEXT-FIGURE 4.—Survival periods of inbred offspring from parents with spontaneous regression of the tumor.



TEXT-FIGURE 5.—Survival percentage of Yoshida sarcoma animals with spontaneous regression of the tumor.

### EXAMPLES OF ABNORMAL PROGRESS

When the tumor cells were transplanted intraperitoneally, they usually proliferated, with an accompanying amount of ascites. The tumor growth infiltrated into the surrounding tissues and the animals succumbed shortly after transplantation. Besides these, there were several animals with an unusual course of tumor growth showing abnormal progress as described in chart 1. Some detailed descriptions of several cases for the past 18 years are given.

1) Nagasaki #177 ♂: Body weight at transplantation was about 80 g.

One ml of the ascitic fluid was transplanted intraperitoneally. For 40 days there was no formation of ascites, but a few tumor nodules formed in the peritoneum and the animal was in an agonized state. The animal was killed and autopsied. There were several tumors in the mesentery, the largest the size of a finger tip, somewhat hard, spherical, and covered with capsules. There were no abnormalities in the omentum, liver, spleen, bone marrow, and lymph glands. There was a small amount of ascites in which tumor cells were present. Histological pictures of tumor infiltration were the same as usual. This was type 3, tumor death after abnormal progress.

2) Sendai #1071 ♂: Body weight at the time of transplantation was 135 g.

The ascites formed after the transplantation was transplanted on the 8th day into another animal and gave a positive result. The animal

became extremely weak 35 days after the transplantation and was killed. One local tumor was found which was transplanted into another animal and produced ascites. This animal belonged to the type 3 tumor death after abnormal progress.

3) Nagasaki #103 ♀: Body weight at transplantation was 80 g.

4) Sendai #1052 ♂: Body weight at transplantation was 120 g.

5) Sendai #1065 ♀: Body weight at transplantation was 100 g.

In these animals, ascites formed after the transplantation and was transplanted into the next generation after 7, 8, and 12 days, respectively, with a positive result. The ascites was also serially transplanted. These animals did not succumb to tumor but lived a normal life. Animal #3 died after 70 days, but there was no tumor infiltration. Animal #4 died after 80 days and #5 after 100 days. Autopsy of these animals revealed no tumor. These animals belong to type 7, spontaneous cure after abnormal progress.

6) Sendai #1084 ♂.

Two ml of ascitic fluid was transplanted; the animal remained normal and died after 50 days. Autopsy revealed a walnut-sized tumor in the omentum, whose center was necrotic. Peripheral portion of the tumor was cut into small pieces and transplanted into 3 rats, 2 of which developed tumor and infiltration, with a small volume of ascites. This tumor was transplanted into another animal and tumor ascites was formed. This rat belongs to type 3, tumor death after abnormal progress.

The abnormal progress observed in commercially procured animals up to March, 1953, when numerous cases were observed, was comparatively few, 202 of 1,724 (12%), but a larger number of abnormal cases could be observed with offspring from spontaneously cured parents (209 of 423, or 50%). These 209 are classified according to chart 1 and shown in table 17. Among them, the following 2 types were observed most frequently. In animals which died with tumor, tumor cells in pure culture disappeared once and then proliferated again (type 2, according to classification in chart 1, 52 animals, 12.3%) to result in tumor death. In those cured, tumor cells in pure culture disappeared and the animals were cured (type 7, according to classification in chart 1, 82 rats, 19.4%).

Some of the findings in particular cases of abnormally progressing tumors are described in table 18.

7) #X109 ♀: Transplanted intraperitoneally from #1738 on May 15, 1950. Body weight at transplantation was 80 g. Tumor cells were growing actively 1 day after transplantation. Numerous acidophils and monocytes were seen in the ascites. Cells were almost in pure culture 2 days after transplantation (referred to as "days later" hereafter). Ascites became thinner 9 days later. Tumors were palpated in the abdomi-

TABLE 17.—Classification of animals\* that died with abnormal progressing tumor and of those in which the tumors regressed

Number of animals	Abnormal progress of tumor growth†							
	1	2	3	4	5	6	7	8
229 (F <sub>1</sub> )	8 (3.5)‡	21 (9.1)	16 (7.0)	3 (1.3)§	0	12 (5.2)	42 (18.3)	5 (2.3)
149 (F <sub>2</sub> )	4 (2.7)	27 (18.1)	4 (2.7)	0	0	11 (7.4)	32 (21.5)	3 (2.0)
45 (F <sub>3</sub> )	1 (2.2)	3 (6.7)	3 (6.7)	0	0	0	8 (17.8)	6 (13.3)
Total	13 (3.1)	51 (12.1)	23 (5.4)	3 (0.7)	0	23 (5.4)	82 (19.4)	14 (3.3)

\*Animals were offspring from resistant parents.

†See chart 1 for definitions of abnormal progress, #1 through 8.

‡Numbers in parentheses indicate percent.

§2) and 3) in chart 1.

TABLE 18.—Transplantation of Yoshida sarcoma cells from animals that died with abnormally progressing tumor

#X109 (130)†	130 days, tumor*	{ #5187 (19) #5188 (24) #5189 (15) #5190 (26)	18 days, ascites	#5191 (11)
#X225 (124)	{ 122 days, ascites* 124 days, tumor*	{ #5199 (21) #5200 (18) #5201 (47)	8 days, ascites	#5205 (13)
			25 days, ascites	{ #5208 (16) #5209 (20)
			26 days, ascites	{ #5210 (19) #5211 (14)
			32 days, ascites	#5212 (19)
			40 days, ascites	#5213 (14)
			42 days, ascites	{ #5214 (11) #5215 (12)
			47 days, tumor of omentum	{ #5216 (10) #5217 (23) #5218 (10) #5219 (16)
#X434 (174)	{ 173 days, ascites* 174 days, tumor* 174 days, heart blood*	{ #5331 (22) #5334 (18) #5335 (38)	19 days, ascites	#2008 (16)
			16 days, ascites	#2019 (12)
			3 days, ascites	#2021 (12)
			4 days, ascites	{ #2027 (15) #2028 (15)

\*Material transplanted.

†Figures in parentheses indicate number of survival days.

nal wall and the omentum 12 days later and tumor cells disappeared. Tumors gradually increased in size to that of a chicken egg, but the size gradually decreased 22 days later and finally the tumors became non-palpable (parturition on Aug. 3; *cf* expt. 30 in table 6 for these offspring). The animal became weak and a tumor the size of a pigeon egg was palpated 127 days later. The animal died on September 22, 130 days later, and the abdomen was opened aseptically. Two tumors of thumb-tip size were found in the mesentery, with grain-sized necrotic foci in the center.

There was almost no abnormality in the omentum and no evidence of pneumonia. One of these tumors was minced and transplanted into 4 animals (*cf* table 18 for results). This is the type 3 tumor death after abnormal progress as classified in chart 1.

8) #X225 ♂: Transplanted intraperitoneally from #4740 on August 27, 1950. Body weight at transplantation was 35 g. Tumor cells were present in the ascites 1 day later, increasing slightly 2 days later. After 4 days the cells disappeared. A grain-sized tumor gradually increasing in size was found on the abdominal wall 15 days later. A similar tumor was found in the right upper wall of the abdomen 85 days later. At this time, tumor cells were not found in the ascites. The animal weakened on December 27 (122 days later) and examination of the ascites indicated a pure culture of tumor cells. The ascites was transplanted into another animal (*cf* table 20 for results). Animal X225 died on December 29 (124 days later). Autopsy showed one tumor the size of a small egg on the right side of the abdominal wall, and a bean-sized one on the left. The former had a parenchymatous surface (about 5 mm thick) with an extensive necrotic lesion in the center. The latter was parenchymatous. Other tumor masses were found: 1 in the upper front paw the size of a sparrow egg, with parenchymatous surface (about 5 mm thick) with a necrotic center, and 3 bean-sized in the right axilla, which were parenchymatous. The ascites was very thick but small in volume. There were no macroscopic changes in the major omentum. Tumor infiltration was observed in the area surrounding the right testis. The parenchymatous portion of the tumor in the upper part of the right front paw was minced and transplanted into 2 animals (*cf* table 18 for results). (Combination of type 2 and type 4 tumor death after abnormal progress according to the classification in chart 1.)

9) #X199 ♂: Transplanted intraperitoneally from #1782 on August 21, 1950. Body weight at transplantation was 60 g. Tumor cells present in the ascites 1 day later gradually increased in number for the next 2 days. Tumor cells in the ascites were in pure culture 6 days later, which continued for 16 days. After 21 days the ascites became quite thin and a grain-sized tumor developed under the skin of the abdominal wall. Tumor cells in the ascites disappeared on September 16 (26 days later). By 58 days a walnut-sized tumor was found on the abdominal wall and a bean-sized one on the left chest. Tumor cells were found in the ascites 66 days

later and the animal died on November 5 (76 days later). Autopsy showed a tumor the size of a chicken egg in the peritoneal wall and a walnut-sized one in the left chest. Both had extensive necrotic foci. The ascites was thick and there was a pure culture of tumor cells with slight infiltration into the major omentum and retroperitoneal tissues. Tumor cells, not transplanted, were present in the heart blood (example of a combination of type 2 and type 4 tumor death after abnormal progress according to the classification in chart 1).

10) #X434 ♂: Transplanted intraperitoneally from #1864 on January 24, 1951. Body weight at the time of transplantation was 75 g. Tumor cells were found in the ascites on January 25 (1 day later), and the cells increased in number 3 days later. The cells in the ascitic fluid were in pure culture 8 days later. The ascites was thin 12 days later. Tumor cells disappeared 20 days later. A small bean-sized tumor appeared on the upper part of the right front paw on June 10, 137 days later, reaching the size of a walnut 167 days later, and a finger tip-sized tumor was palpable in the omentum. The animal became weak on July 16 (173 days later). A few cells, which appeared to be tumor cells, were found in the ascites and the ascites were transplanted into 1 animal (*cf* table 18 for results). The original animal died on July 17 (174 days later). Autopsy showed a walnut-sized tumor in the upper part of the right front paw, three bean-sized tumors in the axilla with numerous necrotic foci, and a parenchymatous tumor the size of a finger tip in the omentum. This was transplanted into 3 animals, 2 of which died the following day, and tumor cells present in the heart blood were transplanted into 1 animal (table 18). Yoshida sarcoma from #X434 was serially transplanted for 24 generations over 8 months, but no abnormality was observed in the subsequent period (combination of type 3 and type 4 tumor death after abnormal progress).

11) #6058 ♂: Transplanted from #1731 on April 30, 1950. Body weight at transplantation was 90 g. The cells in the ascitic fluid were in pure culture 4 days later. The ascites became quite thin on May 15, and a large, bean-sized tumor was found in the abdominal wall. A bean-sized tumor found on the right chest on June 3 grew flat and combined with the tumor in the abdominal wall. Boardlike infiltration developed from the right front paw to the hind paw and walking became difficult. After August 10 the animal gradually recovered and became completely healthy 180 days later. No recurrence was observed. There has been no example of type 5 spontaneous cure after abnormal progress in the group of animals indicated in table 19. The only example of type 5 seen so far was a rat procured from a dealer.

The special abnormal animals described previously were #X225, #X109, and #X434 that died 124, 130, and 174 days after transplantation (table 18). Tumor cells from these 3 were transplanted into other animals. It should be noted here that even the tumor cells from the animals which

TABLE 19.—Comparison of Yoshida sarcoma with ascites hepatoma AH-108 in frequency of formation of tumor in various sites after transplantation of tumor cells into left chamber of the heart

Site	Yoshida sarcoma	Ascites hepatoma	Site	Yoshida sarcoma	Ascites hepatoma
Lymph nodes*	+++†	+++	Sublingual glands	+	+
Heart	+++	+++	Prostate	+	+
Lungs	+++	++	Seminal vesicles	+	—
Kidneys	+++	+	Uterus	+	—
Bone marrow	+++	+	Eyes	—	+++
Liver	+++	+	Skin	—	+
Adrenal glands	++	++	Lacrimal glands	—	+
Ovaries	++	++	Testicles	—	+
Pancreas	++	++	Esophagus	—	+
Thymus	++	+	Epididymis	—	—
Spleen	++	+	Parotid glands	—	—
Stomach	++	—‡	Submaxillary glands	—	—
Intestines	++	—	Tongue	—	—
Brain	+	+++	Bladder	—	—
Skeletal muscles	+	+	Aorta	—	—
Thyroid glands	+	+			

\*In the lymph nodes, the frequency of tumor appearance was almost equal in both cases, but the degree of tumor enlargement was far greater in the ascites hepatoma than in the Yoshida sarcoma.

†+++ Tumors found in more than 8 of 10 rats, macroscopically or microscopically. ++ Tumors found in more than 4 of 10 rats, macroscopically or microscopically. + Tumors found in less than 2 of 10 rats, macroscopically or microscopically.

‡— Indicates that no tumor was found in any of the animals.

had lived over 100 days, with an abnormally progressing tumor growth, made rapid growth when transplanted into a new host and killed the host after normal progression. The animals serving as the first host lived slightly longer than the normal ones but seemed to follow the general trend of transplantation with a fewer number of cells. There was no marked change in the cells themselves, even when the host animal grew abnormally. This rate of growth (malignancy) seemed dependent on the host's condition. The same fact was observed in heterotransplantation of the tumor cells into mice, as will be described later, and its back-transplantation into rats.

#### Autopsy Findings of Tumor Death After Abnormal Progress

Tumor infiltration in animals dying after normal progress appeared macroscopically white or pink, or hemorrhagic, lustrous, with uneven surface, and soft. Histologically, this tumor should be called a "cytoma," in which formation of both connective and silver fibers is few or almost negative.

In type 1 of abnormal progress, the tumor was similar to that in normal progress, but sometimes the degree of growth was much higher. The infiltration was sometimes soft and sometimes hard, and in the latter the connective tissue fibers increased. This was a reaction of the host and may be taken as a kind of resistance phenomenon. There was a strong neutralizing action to the chemical toxic substance produced by tumor

cells, which may have been responsible for host's life despite the growth of tumor.

Tumors of types 3 and 4 of abnormal progress appeared to the naked eye as white or pink, hard, with a smooth surface and a necrotic focus in the center. Parenchymatous cross-section was lustrous. Histologically, the tumor was rich in connective fibers and the surface was covered with a membranous capsule.

In type 2 of abnormal progress, there was a portion rich in cells on the tumor surface which seemed to indicate that tumor cells were freed into the ascites from this portion. This suggests that proliferation of cells in the tumor had been dormant for a certain period after which a rapid growth started in a kind of outburst, causing death of the host.

Types 2, 3, and 4 could be created artificially, such as by the injection of a chemotherapeutic agent. When a life-prolongation effect appeared with cell changes and tissue infiltration, death with such induced tumors was similar to that of types 2, 3, and 4.

As described in abnormal progress of the tumor, animals with tumor cells in the host body for a long period resulted in a strong reaction of the host tissue, especially that of the fibers, and the tumor became fibrous and hard, sometimes with formation of capsules. Cell growth was rather poor. In this case, host reaction appeared morphologically, and there seemed to be a balance of power between tumor cell proliferation and the defense mechanism of the host. At some period, this balance was destroyed and there was an outburst of cell proliferation, resulting in the death of the host.

In types 5 and 6 of the abnormal progress, tumor cells and reactive cells were absorbed and no signs of infiltration remained except slight adhesion.

## PATHOLOGICAL FINDINGS IN ANIMALS WITH TRANSPLANTS OF YOSHIDA SARCOMA

When Yoshida sarcoma was transplanted into the peritoneal cavity of a rat, the cells grew in the ascitic fluid in pure culture and usually infiltrated into the omentum, liver hilum, fatty tissue around sexual organs, lymph nodes, and fatty tissues in the retroperitoneum, mesenteric lymph nodes, and mediastinal lymph nodes. Microscopically, there was invariably an infiltration in the liver, and often metastasis in the lungs (macroscopic hemorrhagic spots), and sometimes in the kidneys. When the sarcoma was transplanted into the peritoneal cavity, the host's survival was usually not more than 10 days, which may be too short a time to observe marked distant metastasis macroscopically.

When the sarcoma was transplanted in the subcutaneous tissue, the progress was slightly longer than that in intraperitoneal transplantation. (This is believed due to a difference in the degree of tissue reaction by transplantation, just described.) In subcutaneous transplantation on the back of a Donryu rat, however, there was no more marked extension

of life than that in intraperitoneal transplantation. Subcutaneous nodules sometimes reached the size of a thumb tip, but most of them were small and some were nonpalpable. Metastases appeared in various places after subcutaneous transplantation, such as in the lymph nodes in the mediastinum retroperitoneum, mesentery, inguinal region and axilla, etc., as well as in the liver, lungs, kidneys, adrenals, sexual organs, and sometimes in the diaphragm, heart, stomach, omentum, and pancreas. When the progress of subcutaneous transplantation took a long time, metastases usually appeared in the organs in the peritoneal cavity, such as the omentum, pancreas, stomach, and intestines. In some cases, tumor cells appeared in the peritoneum and grew in pure culture. In such a case, appearance of tumor growth was similar to that of intraperitoneal transplantation. In subcutaneous transplantation, the liver was only slightly enlarged when the tumor growth took a normal course, but a high degree of cell infiltration could be observed microscopically. Similar infiltration was observed in the spleen.

Such a marked spread of Yoshida sarcoma over the whole body indicated that these tumor cells invaded the blood soon after transplantation and spread through blood vessels. Tumor cells were usually found in smears of peripheral blood of animals in the final stage. It is possible to effect transplantation with blood (5). Early detection of cancer can be made by this method or by collection or cultivation of tumor cells from blood (6, 10). These methods have made it possible to detect tumor cells in the circulating blood, even 12 or 24 hours after intraperitoneal transplantation.

When Yoshida sarcoma was transplanted into the femoral muscle or under the tail skin (lymph space), tumor cells passed through the respective lymph nodes. There was often a systemic metastases when Donryu rats were used.

Kanzaki made a detailed examination of the spread of Yoshida sarcoma over the whole body when the tumor cells were transplanted into the left heart and disseminated through the blood (11). When over  $10^8$  cells were injected into the left heart, though the animal died a few minutes later, tumor cells spread to all the organs examined. The animals did not die rapidly when about  $10^7$  cells were transplanted and tumor death was the usual result. In such a case, there were organs in which the tumor metastasized, and there was definitely a difference in growth rate according to the organs, although a directly transplanted tumor will grow in any place it is transplanted. Table 19 shows a comparison of Yoshida sarcoma and ascites hepatoma AH-108 in the frequency of metastasis and indicates that there is a difference in organs susceptible to tumor formation between these two tumors.

When Yoshida sarcoma was transplanted into the tail vein, femoral vein, or right heart, there was no great difference from the results obtained with transplantation into the left heart. This is probably because the free tumor cells easily pass through pulmonary capillaries, though ascites hepatoma AH-108 showed a high rate of growth in the lungs alone, and only a poor or no growth in other organs.

## HETEROTRANSPLANTATION OF YOSHIDA SARCOMA IN MICE

Yoshida sarcoma cells, if transplanted into the peritoneal cavity of a mouse in over 5 million cells at a time, reached pure culture in 4 days, and the cells decreased rapidly thereafter (12). Back-transplantation of these cells into a rat was possible during this growth period, before the cells disappeared. Mice transplanted with Yoshida sarcoma were all spontaneously cured. If the cells were transplanted into another mouse, at the peak of their growth (the peak determined by peritoneal puncture), Yoshida sarcoma could be serially transplanted and kept alive in mice. As long as Yoshida sarcoma cells were present, back-transplantation into rats was possible. Results of experiments carried out with various strains of mice are given in table 20, which shows that Yoshida sarcoma was kept alive in mice through a maximum of 31 generations of 101 days. The cells were back-transplanted into rats after 101 days and the rats died normally. This indicates that the cells failed to acquire adaptation to mice even after 100 days. The cells did not change and caused no tumor death in mice, as they do in rats. Of about 300 mice used in the whole experiment, 10 or so died (usually, mice do not die after disappearance of tumor cells) and their autopsy revealed the presence of necrosis in the liver, without infiltration of tumor. Some cases showed residual necrotic foci in the pancreas, which is assumed to have resulted from infiltration of the tumor in the pancreas with necrosis remaining after the disappearance of tumor cells. In any case, tumor death in its true sense of the word, such as seen in rats, was never observed in mice.

TABLE 20.—Heterotransplantation of Yoshida sarcoma in mice

Experi- ment No.	Animal	Serial trans- plant gen- eration	Duration (days)	Last positive back-trans- plantation into rats (at day)
1	Swiss	31	101	101
2	German	27	91	91
3	Noninbred white	31	99	99
4	Noninbred white	12	50	47
5	Noninbred white	10	36	25
6	Noninbred white	19	81	39

Yoshida sarcoma resulted from rat cells and it was impossible to transplant it into mice, although it was possible to carry on the cells by serial transplantation in mice by the method shown. Even then, the host mice did not die with tumor in the true sense. Mice will die if the number of Yoshida sarcoma cells transplanted is increased, such as 2 to  $5 \times 10^8$  cells per mouse, in which case the mouse will die of shock through the so-called protein absorption. Even if a mouse were to die, it would be necessary to examine fully whether the death was a direct result of tumor growth. Since the Yoshida sarcoma cells reached pure

culture in the mouse peritoneum at one time, important organs might be affected by the cell growth at this period and the mouse might die of this after disappearance of tumor cells. This is probably the reason for the number of lethal cases and is entirely different from tumor death of rats. In other words, Yoshida sarcoma was produced from rat cells and retained species-specificity.

Yoshida sarcoma cells, in Swiss mice for about 100 days and back-transplanted into rats, did not have a different nature. Serial transplantation of Yoshida sarcoma cells in German mice was carried out for 27 generations of 91 days, and back-transplantation into rats was carried out on the 6th, 9th, 10th, 13th, 16th, 20th, 23d, 31st, 46th, 50th, 52d, 55th, 73d, 75th, 85th, 88th, and 90th day, and rats died by typical infiltration after 8 to 13 days. The last 4 back-transplantations are summarized in table 21, which correspond to attempted transplantation from the cases of abnormal progress described (table 18).

TABLE 21.—Back-transplantation of Yoshida sarcoma from mouse to rat

Serial transplant generation in mice	Total period in mice (days)	Donor mouse No.	Material	Recipient rat No.	Survival (days)
25	85	D 158	4-day ascites	5168	12
26	88	D 159	4-day ascites	5170	10
26	90	D 159	6-day ascites*	5175	24
27	91	D 163	4-day ascites	5180	16

\*The ascites was thin and there were fewer tumor cells.

## CHANGES DURING SERIAL TRANSPLANTATION

In spite of the eventful history in the transplantation of this tumor, Yoshida sarcoma cells have not undergone much morphological change since their discovery (*cf* table 1). Proliferation of a single clone by the transplantation of one single cell has been carried out twice (228th and 426th generation). They also stayed about 1 month in mice (256–262d generation), were preserved in an ice box for 7 days (70th generation), and were obtained from a nodule of an animal 12 to 24 hours after its death (171st generation). The cells were maintained at 60° C for 30 minutes (206th generation), or exposed to a chemical (394th generation). Yoshida sarcoma cells have been passed through Donryu rats since about the 450th generation and some of the results are different from those reported earlier. These results may be due in part to progress in the transplantation technique, but the reason still remains obscure.

### Appearance of Sublines With a Larger Number of Chromosomes

In early days, the chromosome number had been counted in metaphase with Giemsa-stained specimens, but due to fixation with alcohol (metha-

nol), chromosomes failed to separate, and it was difficult to determine their number. Haruo Sato fixed the cells with mercuric chloride and acetic acid and stained them with iron hematoxylin to show the chromosomes. Yoshida sarcoma used for this experiment was a clone obtained by the transplantation of a single cell. According to his report (13), tumor cells had a wide range of chromosome numbers, with 42 as the peak, and cells with V- and J-shaped chromosomes were observed, but such peculiar chromosomes were not necessarily always present. The technique for sketching chromosomes has improved greatly, and it has become possible to separate each chromosome by treatment with hypotonic saline solution, and count the number and examine the shape of the chromosomes. Such examinations were often made with the cells from ascites 4 days after transplantation (though not a clone). Technical progress has narrowed the range of the number of chromosomes, but it still holds that V- and J-shaped chromosomes are not necessarily present at all times (modal number of chromosomes is 40).

Isaka attempted transplantation of a single cell in 1960, using the 920th and 921st generations (14). One of these clones contained large cells which were serially transplanted through more than 100 generations. The cells are still very large (about double the diameter of an ordinary Yoshida sarcoma cell) and the number of chromosomes is also doubled. The drug sensitivity of this clone to Nitromin is the same as that of the parent cells.

#### Changes in Drug Resistance

A detailed report on drug resistance is given in this monograph, but it has been found that some cells acquire natural resistance as transplantation is continued. Cells from a rat of the 906th generation were transplanted into 2 rats of the 907th generation; one was kept in the Sasaki Institute (Dr. Isaka in charge) and the other at the Cancer Chemotherapy Section of the Institute in Nishigahara, Tokyo (Dr. Hiroshi Satoh in charge). These were further serially transplanted and one of the latter of the 919th generation was sent to the Cancer Research Laboratory, Tohoku University, Sendai (Dr. H. Sato in charge). The drug resistance of these 3 strains to Nitromin was found to be different at the end of 1960. The minimum effective dose (MED) values were 1 mg per kg for the tumor strain kept at the Sasaki Institute, and 25 mg per kg for those in Sendai and at the Cancer Chemotherapy Section in Nishigahara. This difference still continues after 100 generations.

In connection with drug resistance, Moriwaki, Imamura, and Sakurai obtained a strain having a very high resistance to Nitromin (15). In its serial transplantation, the tumor-bearing animals had a longer survival than with the usual Yoshida sarcoma (average of 2 days). Although there were no marked differences in the point of infiltration, this tumor strain showed a slight decrease of the so-called malignancy. The strain of Yoshida sarcoma obtained by Dr. Iwao Hirono (16), which was resistant to Nitromin, survived 3 days longer and suggested a decrease

in malignancy, but this strain caused more infiltration into the peritoneal wall than the usual Yoshida sarcoma and seemed to indicate a slight biological difference.

### Fats in the Cell

Appearance of fats in Yoshida sarcoma cells was not marked and could not be detected at the time of active proliferation. Fats appeared only during the degeneration (vacuoles of cells seen in the Giemsa-stained specimen were not due to fats) and there was no fatty change of the nucleus. Fat granules appeared before the death of animals bearing Yoshida sarcoma when the course was normal, probably due to a partial malnutrition from excessive growth of Yoshida sarcoma cells in the peritoneum, which suggested malnutrition of tumor cells. This was similar to the appearance of fat granules 2 to 4 days after transplantation of Yoshida sarcoma into experimentally fasted animals, and probably due to liberation of masked fat by proteolysis inside the cell (fat phanerosis). In this experiment, fat droplets disappeared after 5 days because of utilization of reserve fat in the animal. This resulted in active proliferation of tumor cells. Fats appearing in Yoshida sarcoma cells at the time of natural cure and heterotransplantation into mice coincided with time of the disappearance of degeneration, and this is considered to be fatty degeneration (17).

### Glycogen Granules

Nouchi measured frequency of the appearance of cells with glycogen granules periodically after transplantation of Yoshida sarcoma. The mean frequency was 93 percent 1 hour after transplantation and a maximum of 95 percent after 24 or 48 hours. The granules decreased in number 3 to 4 days later, when the cells attained pure culture, and decreased to 10 percent or lower in the final stage. These tendencies of glycogen granules were the same whether the cells were transplanted on the 2d day or in the final stage, the granules being more numerous in the early stage and less numerous in the final stage. In heterotransplantation of Yoshida sarcoma in mice, the frequency rate reached the maximum of 98 percent 15 to 18 hours after transplantation, with subsequent decrease and a minimum of 23 percent reached at the time of pure culture. Cells with glycogen granules increased thereafter and the value of almost 100 percent was reached just before the disappearance of Yoshida sarcoma cells in the ascites of mice.

At the present, there are very few cells of Yoshida sarcoma with glycogen granules, from the early stage after transplantation to the final stage. However, cells with many glycogen granules appear in a high rate in Yoshida sarcoma drug-resistant strains (granules which disappear by saliva digestion test).

Cells with glycogen granules after administration of an alkylating agent in the susceptible strain of Yoshida sarcoma were reported by Nouchi (18), who showed that the value was almost 100 percent 48 hours

after administration. Cells with these granules decreased when the cells in the peritoneum showed recovery. The same results are now being obtained.

## SUMMARY

Yoshida sarcoma, an ascites tumor of rat, was discovered in 1943 and has passed through 1,000 generations for the past 18 years by transplantation of cells from the ascitic fluid into the rat of a next generation. During this time, this sarcoma has been used as material for numerous tumor experiments and is being used to a great extent in Japan. The record of these transplantations has now been put in order.

Transplantability of Yoshida sarcoma is very high and the tumor usually takes the so-called "normal course." The tumor cells in the ascitic fluid reach a pure culture 2 to 3 days after transplantation, and the abdomen of transplanted animals becomes distended due to the increased volume of the ascites. The animals usually die after 7 to 10 days on an average. In some cases, tumor "take" is poor and its growth takes the so-called "abnormal course." Sometimes spontaneous cure of the tumor occurs and only a small percent becomes nontransplantable. The course of progress of the tumor was examined in detail with many animals. Examination was also made on the transplantability of Yoshida sarcoma in the litters obtained from parent animals in which transplantation failed. These experiments have suggested the presence of a genetic factor(s) that takes part in the transplantability of Yoshida sarcoma. Yoshida sarcoma originated in a noninbred Japanese white rat and similar noninbred rats were used for subsequent transplantation. Since 1954, however, a colony of animals called Donryu rats has been used. This animal strain shows a high and stable transplantability of Yoshida sarcoma. By the use of these animals, transplantability rate is almost 100 percent and survival of the animals is constant at 7 to 8 days, only a few showing abnormal progressing tumors.

Heterotransplantation of Yoshida sarcoma into mice showed no change in the nature of these tumor cells. Transplantation was also made in rats of various strains. Some of these strains showed transitory cell growth, but a complete regression occurred and some of the animals were cured spontaneously, as in heterotransplantation.

Malignancy of a tumor can be expressed by the rate of tumor growth and transplantability rate, but there is the problem of host-tumor relationship. For cancer to show its fundamental nature—the malignant growth—it is necessary that there be optimal conditions for such growth, and it does not follow that cancerous growth appears under every circumstance and at any time. This nature becomes latent under adverse or unsuitable conditions. The mean survival time can be prolonged or spontaneous cure (decreased transplantability) can be increased by the physical state of the host. These conditions differ according to the strains of rats, and suggest some genetical factor(s) involved.

On the other hand, in abnormal progress, the tumor cells are present over a long period without apparent growth but begin active growth almost suddenly at a certain period to infiltrate the tissue. This seems to break the balance kept up to that time and the host animal dies. In human beings, sudden progress of cancer infiltration is sometimes seen and this is known as the "outburst." In such cases, the phenomenon is generally accepted as the accelerated growth of cancer cells (acceleration of malignancy) but, from the result of animal experiments, this is frequently due to changes in the condition of the human host rather than changes in tumor cells. Mammary and prostate cancer are examples in which conditions of cancer cells and the host are kept in good balance, or in which cancer is under the control of the conditions of the host and environment. These cancers are controlled by hormonal conditions and are considered different from the real cancer which shows autonomous proliferation. However, it seems more appropriate to consider that we have not as yet discovered the substances in most of the usual cancer cases which correspond to hormones for mammary and prostate cancers.

If cancer were to be considered as carrying out its own life inside the host's body—like protozoa—it could be handled as a parasite, which would give the basis for cancer chemotherapy. Chemotherapy is a method of severing a parasitic organism without injuring the host. Chemotherapy of cancer has decreased the growth rate and also the number of cancer cells. However, some of these cells are resistant to chemicals. This resistance is acquired by some cancer cells, but the cells are usually provided with their own natural resistance (90% of ascites hepatoma are of the resistant type). There is a limit to the lowering of malignancy of cancer cells themselves. Abnormal progression of tumor growth and heterotransplantation decrease the malignancy of cancer cells. The problem in cancer is related to its malignancy, and study of decreased malignancy leads to the direction of cancer treatment.

## REFERENCES

- (1) YOSHIDA, T., MUTA, Y., and SASAKI, Z.: Studien über das "Ascites-Sarcoma" (I). Proc Imp Acad (Japan) 20: 611-616, 1944.
- (2) YOSHIDA, T.: The Yoshida sarcoma, an ascites tumor. Gann 40: 1-20, 1949.
- (3) ———: Studies on an ascites (reticuloendothelial cell) sarcoma of the rat. J Nat Cancer Inst 12: 947-969, 1952.
- (4) ISHIBASHI, K.: Studies on the number of cells necessary for transplantation of Yoshida sarcoma. (Transmission of tumor with single cell.) Gann 41: 1-14, 1950.
- (5) KURATA, T.: Haematogenous dissemination of tumor cells. Trans Soc Path Jap 48: 1329-1337, 1959.
- (6) SATO, H. *et al.*: Studies on the *in-vitro* growth of a small number of Yoshida sarcoma cells. Sci Rep Res Inst Tohoku Univ.-C, 11: 1-11, 1962.
- (7) HOSOKAWA, K.: Studies on transplantation of tumors with a single cell. Experiments with the Yoshida sarcoma and ascites hepatoma. Tohoku Igaku Zasshi 47: 557-568, 1953.

- (8) KAZIWARA, K.: Complete and incomplete regression of the Yoshida sarcoma and their relations to hereditary constitution of rats. *Gann* 43: 242-245, 1952.
- (9) SATO, H.: Intraperitoneal transplantation of the Yoshida ascites sarcoma and the ascites hepatoma to various American strains of rats. *J Nat Cancer Inst* 15: 1367-1378, 1955.
- (10) ———: Cancer cells in the circulating blood with reference to cancer metastasis. *Bull WHO* 26: 675-681, 1962.
- (11) KANZAKI, K.: Distribution of tumors in various organs following the transplantation into left chamber of heart (Studies with Yoshida sarcoma and ascites hepatoma). *Gann* 44: 445-461, 1953.
- (12) ATSUMI, A., and HOSOKAWA, K.: Inherent and acquired resistance of mice against Yoshida sarcoma cells. *Gann* 43: 240-242, 1952.
- (13) SATO, H.: On the chromosomes of Yoshida sarcoma. Studies with tumor cells proliferated in the peritoneal cavity of rat transplanted with single cell. *Gann* 43: 1-15, 1952.
- (14) ISAKA, H., SATOH, H., OOSHII, Y., and IZUMITANI, M.: Chromosomal features in the Yoshida sarcoma and its two different derivations, polyploid and  $HN_2$ -resistant sublines. *Gann* 55: 163-174, 1964.
- (15) ISHIDATE, M., SAKURAI, Y., MORIWAKI, A., and IMAMURA, H.: Studies on carcinostatic substances (XX). Studies on the culture of Yoshida sarcoma cells. *Chem Pharm Bull* 7: 690-694, 1959.
- (16) HIRONO, I.: Some properties of Yoshida sarcoma cells resistant to methyl-bis-( $\beta$ -chloroethyl)-amine N-oxide. *Proc Soc Exp Biol Med* 88: 147-149, 1955.
- (17) ASANO, T.: On the fat substance of Yoshida sarcoma cells. *Tohoku Igakusassi* 47: 569-575, 1953.
- (18) NOUCHI, F.: Glycogen of tumor cells. I. Experimental studies on the Yoshida sarcoma cells. *Fukushima Med J* 11: 619-641, 1961.



## Establishment of Ascites Hepatomas in the Rat, 1951-1962<sup>1</sup>

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ASCITES hepatomas in rats were reported for the first time in 1951 by Yoshida, Sato, and Aruji (24), of the Pathology Department of Tohoku University, Sendai, Japan. Further studies on ascites hepatomas were carried out at the Sasaki Institute in Tokyo, and many reports (3-8, 10-12, 18, 19, 22, 23) published by that Institute on fundamental problems in cancer research were based on the study of ascites hepatomas. Strains of these hepatomas have been distributed to other investigators for experimental cancer studies. Before describing the establishment of ascites hepatomas, we will discuss their value in experimental cancer.

The ascites hepatoma may be defined as an ascites tumor originating from the epithelial cells of the liver of an animal.

The term "ascites tumor" is often used by cancer investigators. Characteristics common to all ascites forms of transplantable tumors are: 1) The inoculated tumor cells can proliferate in ascitic fluid as a single cell or as a cell cluster and produce tumorous ascites. 2) Serial transplantation in animals and other cancer experiments in specific fields of study can be carried out with this tumorous ascites.

Morphologically, the fundamental difference between ascites tumors and solid transplantable tumors is that the former do not require morphous stromata such as blood vessels, and connective tissues in order to proliferate. They can proliferate not only in ascitic fluid but also in fluids in the pleural and cerebrospinal cavities. Therefore, these tumors have been described for the first time as "free cell tumors (cancer)" by Goldie (2) and as "fluid tumors (cancer)" by Yoshida (20, 21). They were so designated to distinguish them from other transplantable tumors generally called "solid tumors."

However, there is no essential difference between "solid tumors" and intraperitoneally inoculated "ascites tumors," except that the former need morphous stroma to proliferate. When ascites tumors are inoculated subcutaneously, they form tumor nodules containing blood vessels and connective tissues. Even when transplanted intraperitoneally, some infiltrating tumor nodules containing morphous stroma are found in the omentum, mesenterium, peritoneum, and

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diaphragm. In the very early stages of nodular tumor development with metastases, it is probable that a few cancer cells grow in the tissue fluid without any kind of morphous stroma. In peritoneal metastases of carcinoma in human beings, growing tumor cells can sometimes be detected in the ascitic fluid, and the existence of morphous stroma is not a *sine qua non* for the growth of these cells.

Thus, the difference between nodules and ascites tumors is a matter of degree, and the ascites tumor can be considered as a "model" of cancer which needs minimum requirements for growth and proliferation.

Twenty-three different strains of ascites tumors from Japan and abroad are listed in tables 1 and 2. Each tumor has specific characteristics as well as characteristics in common with ascites tumors, and each one is

TABLE 1.—Principal ascites tumors established before 1951

Strain	Primary tumor	Originated in	Animal	Converted in	Reported by
Flexner-Jobling carcinoma	"Gemischtzelliges" Sarkom (Karzinom?)	1906	Rat	1906	Hesse
Ehrlich ascites tumor	Mammary cancer	?	Mouse	1930	Löwenthal
Yoshida sarcoma	Reticuloendothelial cell(?) sarcoma	1943	Rat	1943	Yoshida
Sarcoma 37	Carcinoma	1906	Mouse	1950	Goldie
Lymphosarcoma 6C3HED	Lymphosarcoma	1941	Mouse	1950	Klein
Rhabdomyosarcoma MC1A	Rhabdomyosarcoma	1945	Mouse	1950	Klein
Krebs 2 carcinoma	Carcinoma simplex	1933	Mouse	1951	Klein
Sarcoma 180 (Crocker)	Polymorphous cell sarcoma	1941	Mouse	1952	Goldie

TABLE 2.—Principal ascites tumors reported in Japan

Strain	Primary tumor	Originated in	Animal	Converted in	Reported by
MTK-I	Sarcoma	1951	Rat	1951	Makino
MTK-II	Sarcoma	1951	Rat	1951	Makino
MTK-III	Sarcoma	1951	Rat	1951	Makino
Takeda sarcoma	Spindle cell sarcoma	1951	Rat	1951	Takeda
Hirosaki sarcoma	Lymphosarcoma	1951	Rat	1951	Usubuchi
Usubuchi sarcoma	Spindle cell sarcoma	1952	Rat	1952	Usubuchi
Formalin sarcoma	Round cell sarcoma	1952	Rat	1952	Watanabe
MH 134	Hepatoma	1952	Mouse(C3H)	1953	Sato
MH 129	Hepatoma	1952	Mouse(C3H)	1953	Sato
MH 129P	Hepatoma	1952	Mouse(C3H)	1954	Sato
MH 129F	Hepatoma	1952	Mouse(C3H)	1954	Sato
Lymphosarcoma tumor	Lymphosarcoma	1954	Mouse(NA2)	1954	Nishizuka
Watanabe ascites tumor	Hepatoma	1954	Rat	1954	Watanabe
SN 36	Leukemic lymphosarcoma	1955	Mouse(DD)	1955	Nakamura
Yamagata sarcoma	Spindle cell sarcoma	1956	Rat	1956	Atsumi

widely used for cancer studies. However, for use in transplantation experiments, it is essential that the tumors can be compared at any time with normal tissue, or the cells from which they originated, and that the tumors can be compared with other tumors which originated from cells of common ancestry.

It is difficult to determine whether the 23 different strains shown in tables 1 and 2 satisfy the two foregoing conditions. For example, Ehrlich ascites carcinoma was derived from mammary carcinoma in mice, maintained by subcutaneous transplantation for generations, and then converted to the ascites form in 1930 by Löwenthal and Jahn. At the present time, it is difficult to determine the epithelial characteristics of the Ehrlich tumor. Sarcoma 37 was so designated after first being grown as a carcinoma and then being converted to the ascites form in 1950 by Goldie (2). The ancestral cells of Yoshida sarcoma are assumed to be reticuloendothelial (21), but there is no definite proof. Thus, comparative studies of Yoshida sarcoma and other strains of tumors derived from common cells, as are Yoshida sarcomas, cannot be carried out. The other 20 strains listed in tables 1 and 2 are also unsatisfactory.

Only ascites hepatoma strains satisfy the two previously mentioned conditions. The first ascites hepatoma was established in 1951. Since then 59 different strains of ascites hepatomas have been reported (4, 12, 15, 25, 26-28). All were derived from azo dye-induced cancer of the liver in rats. The ascites hepatoma seems to be the most valuable transplantable tumor in ascites form used in cancer research.

## ESTABLISHMENT OF ASCITES HEPATOMA IN THE RAT

The first ascites hepatoma was designated as strain AH 137 (17). Of the 59 different strains of rat ascites hepatomas established from 1951-61, 54 have been maintained in our laboratory by serial transplantations in animals. Five strains were lost: AH 137 and AH 108 were discontinued for reasons of economy; AH 27, AH 364, and AH 98A were lost because of technical failure during inoculations. Data concerning 56 primary tumors from which strains of ascites hepatomas were derived are summarized in table 3.

All strains of ascites hepatomas have a designation preceded by the letters AH. The number following is that given to the primary tumor animal during the time of feeding of the carcinogen and has no other special significance. To each of the strains established after 1961, A, B, or C was added. A, B, and C indicate derivation from primary tumor ascites, from the largest primary tumor nodule, and from the second largest primary tumor nodule, respectively. All strains derived from the same rat are designated by the same number.

Ascites hepatomas are established by using primary tumor ascites or tumor nodules as inoculum. The results of transplantation are in-

TABLE 3.—History of original tumor animals

Strain (AH)	Year established	Carcinogen used	Months of feeding	Strain of rat*	Experiment (days)	Inoculum			Histological type of tumor†	Generation in which converted
						A†	B†	C†		
1	1951	DAB	?	a	?	x			?	1
2	1952	DAB	?	a	?		(x)		?	1
3	7974	DAB	6	a	f		(x)		?	1
4	601	AAT	6	a	f				?	2
5	602	AAT	3	a	f	x			?	1
6	66	DAB	3	a	m	x			?	1
7	63	"	4	a	f	x			(I + II)	1
8	149	"	4	a	m	x			?	1
9	39	"	4	a	f				?	1
10	49	"	4	a	f		(x)		(I + II + III)	9
11	99	Derived from AH	66	a	m		(x)		(I + II + III)	1
12	322	DAB	7	a	m	x			(I + II + III)	1
13	414	"	6	a	m	x			(I + II + III)	1
14	21	"	8	a	f		(x)		(I + II + III)	1
15	318	"	7	a	m		(x)		(III)	1
16	423	"	5	a	f	x			(I + II)	1
17	13	"	7	a	f		(x)		(I + II)	2
18	62	"	7	a	m		(x)		(I + II)	1
19	173	"	6	a	m	x			(I)	1
20	408	"	5	a	m	x			(III)	1
21	310	"	5	a	m	x			(I + II)	1
22	311	"	5	a	m	x			(I + IV)	1
23	272	"	5	a	m	x			(I + II)	1
24	286	"	5	a	m	x			(I + II)	1
25	127	"	5	a	m		(x)		?	1
26	62F	Derived from AH	62	a	m				?	1
27	44	DAB	5	a	m	x			?	1

28	34	1960	DAB	5	a	m	?	x	?	1
29	41A	"	3'MeDAB	6	b	m	180	x	180	1
30	41B	"	"	6	b	m	180	x	180	1
31	41C	"	"	6	b	m	180	x	180	1
32	131A	"	"	3	b	m	186	x	186	1
33	131B	"	"	3	b	m	186	x	186	1
34	108A	"	"	4	b	m	188	x	188	1
35	98A	"	"	4	b	m	177	x	177	1
36	84A	"	"	5	b	m	188	x	188	1
37	84B	"	"	5	b	m	188	x	188	1
38	57B	"	"	5	b	m	174	x	174	2
39	122A	1961	"	3	b	m	221	x	221	2
40	122B	"	"	3	b	m	221	x	221	2
41	42B	"	"	6	b	m	178	x	178	1
42	61B	"	"	5	b	m	176	x	176	2
43	70B	"	"	5	b	m	229	x	229	3
44	65C	"	"	5	b	m	219	x	219	4
45	55A	"	"	6	b	m	195	x	195	1
46	107B	"	"	4	b	m	195	x	195	5
47	60C	"	"	6	b	m	180	x	180	8
48	106B	"	"	4	b	m	326	x	326	9
49	136B	"	"	3	b	m	325	x	325	9
50	100B	"	"	4	b	m	354	x	354	3
51	109A	"	DAB	5, 5	b	m	262	x	262	14
52	143A	"	"	"	b	m	317	x	317	1
53	150A	"	"	"	b	m	213	x	213	1
54	225A	"	"	"	b	m	230	x	230	1
55	371A	"	"	"	b	m	235	x	235	1
56	210A	"	"	"	b	m		x		1

\*a: Hybrid rat. b: Donryu rat.

†A: primary tumor ascites. B: largest tumor nodule. C: second largest tumor nodule. (x): Several tumor nodules in the liver and peritoneal tissues.

‡I: Hepatoma; II: intermediate type of I and III; III: cholangiocarcinoma; IV: carcinoma simplex.

Courtesy Nagasaki Igakka Zasshi (Nagasaki).

fluenced by various conditions, and not all primary tumor ascites and tumor nodules can be continued or converted to ascites form. The balance between the characteristics and number of inoculated cells and the sensitivity of the host animals to the inoculated tumor cells may be positive or negative. In positive cases, unknown factors in the inoculated cells might determine their ability to proliferate in ascitic fluid.

Results obtained through 1961 show that the rate of conversion into ascites form apparently depends on the homogeneity of the rats employed. Results obtained before and after 1961 will be compared later.

Before 1961, hybrid Japanese albino rats were used, thereafter Donryu rats, a highly susceptible, closed colony of our own breed, were used in carcinogenesis and transplantation experiments.

#### Development of Ascites Hepatomas from Primary Tumor Ascites

In 145 of 416 (30%) hybrid rats with primary tumors induced by azo dyes, primary tumor ascites were detected when animals were in their terminal stages. As shown in tables 4 and 5, 19 of 113 (17%) showed positive transplantation in hybrid animals (24, 26-28); in 15 of 113 rats (13%) it was converted into ascites form. On the other hand, with Donryu rats, 14 of 32 (44%) were positive and 13 of 32 (41%) proliferated into the ascites form (14). The percentage of positives for Donryu rats was about 3 times as high as for hybrids.

TABLE 4.—Transplantation rate for primary tumor ascites and nodules

Rat	Inoculum		Nodules	
	Ascites			
Hybrid	19/113	17%	57/174	33%
Donryu	14/32	44%	65/119	55%

TABLE 5.—Rate of establishment of ascites hepatoma from primary tumor ascites and nodules

Rat	Inoculum		Nodules	
	Ascites			
Hybrid	15/113	13%	14/174	8%
Donryu	13/32	41%	16/119	13%

Although the transplantation rate with primary tumor nodules and primary tumor ascites was about the same, it is difficult to compare the results because of the difference in the number of tumor cells inoculated into the host. In the transplantation of the primary tumor ascites, the number of tumor cells was about 50 to 100 cells per ml, and the volume of the inoculated tumor ascites was usually less than 10 ml; in nodule transplantation about  $5 \times 10^7$  tumor cells were used.

Even with established ascites tumor strains, the results are not constant with a small number of tumor cells. Table 6 shows the correlation be-

tween the transplantability of primary tumor ascites and the number of proliferating tumor cells. When many tumor cells were detected, higher transplantability was shown: 43 percent (3/7) in hybrid rats and 70 percent (7/10) in Donryu rats. Thus, in transplantation with primary tumor ascites containing numerous proliferating tumor cells, a higher degree of transplantability may be expected than in transplantation with primary tumor nodules.

TABLE 6.—Intraperitoneal transplantation with primary tumor ascites

Number of cells in 1 drop	1958-60		1960-61		Volume of inoculum per rat					
					<1 ml		1~5 ml		>5 ml	
< 5	1/16	6%	4/11	36%	0/1	0%	3/8	38%	1/2	50%
<10	2/28	7%	2/10	20%	0/6	0%	0/2	0%	2/2	100%
<50	1/6	17%	2/4	50%	0/2	0%	1/1	100%	1/1	100%
>50	3/7	43%	5/6	83%	1/1	100%	2/3	67%	2/2	100%
Total	7/57	12%	13/31	42%	1/10	10%	6/14	43%	6/7	86%

Courtesy Nagasaki Igakkai Zassi (Nagasaki).

Why the rate of transplantation depends on the number of tumor cells inoculated, even in established transplantable strains, is quite difficult to explain, but it seems to be a problem worth further study.

In some rats inoculated with tumor ascites, tumor nodules were found but no further development of tumor ascites was observed. These cases seem to have an important bearing on the problem of ascites conversion, but no conclusions have as yet been reached.

In positive transplantations, the tumors cells began to appear at 7 to 10 days after inoculation and gradually increased in number. The animals died 3 or 4 weeks after transplantation. The medium survival time in days for the first generation host was usually longer than for later generations.

In a few instances, the inoculated cancer cells grew at first, showed a pure culture of cancer cells in ascitic fluid, and then disappeared rapidly. The animals generally survived, but transplantations made with such pure cultures usually gave negative results. However, occasionally an ascites hepatoma was established from such transplantations (AH 55A) (12). This phenomenon could not be pursued.

#### Establishment of Ascites Hepatomas From Primary Tumor Nodules

The procedure for the transplantation of the tumor nodules was as follows: Animals with primary azo dye-induced tumors were killed in the terminal stage under ether narcosis. The primary tumor nodule was removed aseptically. A minced tumor gruel containing a small amount of antibiotics was injected intraperitoneally into new hosts. In the pre-1961 experiments with hybrid rats, primary tumor nodules from the liver and infiltrating or metastatic tumor nodules from peritoneal tissues were mixed and crushed in a petri dish, and the gruel was used for trans-

plantation. The volume inoculated in each host was 0.1 to 2.0 ml. In experiments with Donryu rats, the largest and the next largest tumor nodules were removed; each served as an inoculum. The volume inoculated in each host was 0.5 ml. The number of the tumor cells transplanted was about  $5 \times 10^7$ .

The following results were obtained with hybrid rats (4, 26-28). Of a total of 174 tumor nodules transplanted, 57 (33%) took; 14 (8%) of these were converted into the ascites form. With Donryu rats, 65 (55%) of 119 primary tumor nodules gave positive transplants; 16 (13%) of these were converted into the ascites form (12, 14). The data for each experimental year are shown in table 7.

TABLE 7.—Transplantation with primary tumor nodules

Year	Carcinogen	Number of primary tumor animals	Number of nodules intra-peritoneally transplanted	Results						Reported by
				Positive				Negative		
				Ascites		Nodular				
					%	%	%	%	%	
1951	DAB	25	34	4	12	10	29	20	59	Aruji
1952	AAT	19	14	1	7	2	14	11	78	Isaka
1956	DAB	29	8	1	13	4	50	3	38	Nakamura <i>et al.</i>
1957	DAB	169	72	5	7	18	25	49	68	Odashima <i>et al.</i>
1958	DAB	76	38	1	3	7	18	30	79	Odashima <i>et al.</i>
1960	DAB	?	8	2	25	2	25	4	50	Odashima <i>et al.</i>
1961	3'MeDAB	83	A83*	13	16	41	49	29	35	Odashima
			B36†	3	8	8	22	25	70	

\*A: the largest tumor nodule.

†B: the second largest tumor nodule.

Often, some pieces of the inoculated tumor tissue remained in the ascitic fluid for 5 to 20 days. Central necrosis with accompanying monocytes showing phagocytosis and many basophilic leukocytes were observed. When positive transplantation occurred in ascites form, cancer cells began to appear increasingly after 7 to 10 days, but occasionally tumor cells could first be detected 3 to 4 weeks after transplantation.

In some instances, many dividing tumor cells were seen initially in the ascitic fluid, but these disappeared rapidly and the animals survived. These cases were similar to some in which primary tumor ascites were inoculated.

Occasionally, when tumor cells were probed for in the ascitic fluid, small tissue was taken from tumor nodules which had formed on the peritoneal wall. These tissues often contained nonepithelial elements. Even when no nonepithelial tissues were present, the surface of the tissue was not as smooth as that of tumor islands.

The number of the transplant generation of ascites hepatoma is determined as follows: A tumor of a given transplant generation in which tumor cells proliferated in a pure culture, and from which serial intra-

peritoneal transplantations with tumor ascites could be followed for long periods, was designated as the first generation of the ascites hepatoma. When the first generation receiving the primary tumor was the first transplant generation of the ascites hepatoma, a "direct conversion or direct establishment" was designated. On the other hand, when there was no conversion into ascites form until a later generation, "indirect conversion or establishment" was designated.

### *Discussion*

Ascites hepatomas were produced in about 13 percent of hybrid rats in which the primary tumor ascites was transplanted, and in about 8 percent in which the primary tumor nodules were transplanted. With Donryu rats, the percentages of takes were 41 and 13 percent, respectively. Transplantation with azo dye-induced liver cancers showed 3 patterns—ascites positive, nodular positive, and negatives. The reason why some hepatomas grow as the ascites and others as the nodular type is obscure. Serial experiments carried in an attempt to resolve this problem are described.

### Comparison of Transplantability of Primary Tumor Ascites and Primary Tumor Nodules

The transplantation rate for primary tumor ascites was 17 percent (19/113) with hybrid rats and 44 percent (14/32) with Donryu rats, while that for primary tumor nodules was 33 percent (57/174) and 55 percent (65/119), respectively. The ascites conversion rate was 13 percent (15/113) with hybrid rats and 41 percent (13/32) with Donryu rats by inoculation with primary tumor ascites, and 8 percent (14/174) with hybrid rats and 13 percent (16/119) with Donryu rats by inoculation with primary tumor nodules (tables 4 and 5). Thus, the transplantation rate for tumor nodules was relatively higher than for tumor ascites. Yet the rate of conversion into ascites form was higher in the latter cases than in the former. As previously shown, the successful transplantation of tumors depends on the number of cells inoculated; with a sufficiently large number of tumor cells proliferating in the ascites, the possibility of success is greater. With the same number of tumor cells, the transplantation is usually more successful with primary tumor ascites than with primary tumor nodules.

It seems that primary tumors which produce primary tumor ascites can proliferate without any morphous stroma or cellular independence; therefore, the developing tumor cells possess a higher degree of autonomy. Since such tumor cells showed a higher degree of transplantability, it may be said that transplantability and autonomy are closely related.

The results of transplantation of primary tumor nodules taken from Donryu rats (14) with or without primary tumor ascites are presented in table 8. The transplantability of primary tumor nodules from animals

in which primary tumor ascites occurred was greater than from animals without primary tumor ascites. In the former, the rate of transplantation was 75 percent (42% occurred in ascites form); in the latter it was 66 percent (10% occurred in ascites form). Tumor nodules associated with primary tumor ascites showed a convertibility into ascites tumors about 4 times greater than nodules not associated with primary tumor ascites.

TABLE 8.—Transplantability of primary tumor nodules from animals with tumorous and nontumorous ascites

Primary tumor ascites	Num- ber of nodules trans- planted intra- peri- tone- ally	Positive				Negative	
		Ascites		Nodular			
Positive	24	10	42%	8	33%	6	25%
Negative	59	6	10%	33	56%	20	34%

Primary tumor ascites was observed in less than 30 percent of animals with azo dye-induced tumors; 10 percent had a large number of cells in the fluid. Tumor nodules from all the animals could be used to establish ascites hepatoma by serial transplantation. Thus, both primary tumor ascites and nodules may be used for inoculations.

Tumor nodules of various sizes and characteristics are often found in animals with azo dye-induced tumors. A number should be used to study the correlation between transplantability and the histological pattern of induced liver cancer.

#### Correlation Between Convertibility Into Ascites Form and the Kinds and Doses of Carcinogen Given to Primary Tumor Animals

Carcinogens given to rats in a series of experiments were 0.1 to 1.0 percent *o*-aminoazotoluene (AAT) (4), 0.06 percent 4-dimethylaminoazobenzene (25-28), and 0.05 percent 3'-methyl-4-aminoazobenzene (3'MeDAB) (12).<sup>4</sup> The transplantability of the primary tumor ascites and tumor nodules which developed in the animals by the administration of these substances is shown in table 9. When primary tumor nodules were used for transplantation, 55 percent given 3'MeDAB, 34 percent given DAB, and 21 percent AAT had positive transplants. But when primary tumor ascites was used for transplantation, 44 percent had positive transplants in the 3'MeDAB group, 16 percent in the DAB group, and 0 percent in the AAT group. In both, the transplantability of these tissues was roughly proportional to the carcinogenic strength of the substances given to the original animals. Because the animals in the 3'MeDAB group were Donryu rats and those in the other two groups

<sup>4</sup> *Chemical Abstracts'* nomenclature: *o*-aminoazotoluene = 4-*o*-tolylazo-*o*-toluidine; 4-dimethylaminoazobenzene = *N,N*-dimethyl-*p*-phenylazoaniline; 3'-methyl-4-aminoazobenzene = *N,N*-dimethyl-*p*-(*m*-tolylazo)aniline.

TABLE 9.—Transplantability of primary tumor ascites and nodules from rats with AAT, DAB, and 3'MeDAB

Inoculum	Carcinogen	Number of rats	Results					
			Positive				Negative	
			Ascites		Nodular			
			( $\%$ )		( $\%$ )		( $\%$ )	
Nodules	AAT	14	1	7	2	14	11	79
	DAB	160	13	8	41	26	106	66
	3'MeDAB	119	16	13	49	41	54	45
Ascites	AAT	7	0	0	0	0	7	100
	DAB	106	15	14	4	4	89	84
	3'MeDAB	32	13	41	1	3	18	56

were hybrids, the correlation in convertibility could not be analyzed completely.

The correlation between the transplantability of primary tumors and the dose of carcinogen in the 3'MeDAB group is shown in tables 10 through 13 (14). The experimental procedures were as follows: Six-week-old Donryu male rats, 4 groups of 30 each, were given 0.05 percent 3'MeDAB in the diet for 3, 4, 5, and 6 months, respectively, after which they were given the basic diet. Each liver cancer was transplanted into the peritoneal cavity of a normal Donryu rat and the transplantability was compared. Table 10 shows the transplantability for primary tumor ascites, table 11 for primary tumor nodules, and table 12 for each rat with primary tumor. The group transplanted with primary tumor ascites was so small that no comparison could be made (table 10). On the other hand, the transplantability of the largest tumor nodule for each animal was 74 percent in group IV, 67 percent in group III, 67 percent in group II, and 54 percent in group I, and that of the second largest nodule was 37 percent in group IV, 33 percent in group III, and 25 percent in group II (table 11). This shows that the transplantability of the primary tumor nodules was proportional to the period of carcinogen administration. The transplantation rate with primary tumors was 78, 72, 67, and 54 percent in groups IV, III, II, and I, respectively (table 12).

TABLE 10.—Results of transplantation with primary tumor ascites

Group	Months of feeding	Rats inocu- lated	Successful transplantations					
			Failed		1 to 5 generations		More than 6 generations	
					Number	Percent	Number	Percent
I	3	5	3	60	0	0	2	40
II	4	4	2	50	1	25	1	25
III	5	7	6	86	0	0	1	14
IV	6	7	5	71	0	0	2	29

TABLE 11.—Results of transplantation with the largest and the second largest tumor nodules

Group	Months of feeding	Size of tumors	Number of rats	Successful transplantations					
				Failed		1 to 5 generations		More than 6 generations	
				Number	Percent	Number	Percent	Number	Percent
I	3	Largest	13	6	46	3	23	4	31
		Second largest	0	—	—	—	—	—	—
II	4	Largest	18	6	33	6	33	6	33
		Second largest	8	6	75	1	13	1	13
III	5	Largest	25	9	36	8	32	8	32
		Second largest	12	8	67	1	8	3	25
IV	6	Largest	27	7	26	8	30	12	44
		Second largest	16	10	63	1	6	5	31

Courtesy Gann (Tokyo).

TABLE 12.—Results of transplantation of liver cancers induced in rats treated with 3'MeDAB for 3 to 6 months \*

Group	Months of feeding	Number of nodules intraperitoneally transplanted	Successful transplantations					
			Failed		1 to 5 generations		More than 6 generations	
			Number	Percent	Number	Percent	Number	Percent
I	3	13	6	46	3	23	4	31
II	4	18	6	33	6	33	6	33
III	5	25	7	28	9	36	9	36
IV	6	27	6	22	7	26	14	52

\* Sometimes in the same rat, only one tumor nodule could be transplanted; another transplantation failed.

Courtesy Gann (Tokyo).

From the results of these experiments, the transplantability of the largest and the second largest tumor nodules from each rat was examined. As shown in table 13, the transplantability for the largest tumor nodule was 67 percent (24/36) and that for the second largest one was 33 percent (12/36).

Interpretation of the foregoing results is very difficult, but the following speculations may be made. 1) Longer periods of azo dye-feeding might result in the development and proliferation of more tumor cells because there might be more chance for transplantable cancer cells to develop. However, the characteristics of the cancer cells might not depend on the dose administered, but might be influenced by other factors. 2) The malignant character of cancer cells which appeared after a short period of administration of carcinogens might be changed by subsequent administration, after a longer period of azo dye-feeding, and cells with higher transplantability might develop. 3) The malignant character of a cancer

TABLE 13.—Results of transplantation with different sized tumor nodules from the same rat

Group	Months of feeding	Num- bers of rats	Size of tumors	Successful transplantations					
				Failed		1 to 5 generations		More than 6 generations	
				Num- ber	Per- cent	Num- ber	Per- cent	Num- ber	Per- cent
II	4	8	Largest	4	50	2	25	2	25
			Second Largest	6	75	1	12	1	12
III	5	12	Largest	5	42	5	42	2	17
			Second Largest	8	67	1	8	3	25
VI	6	16	Largest	3	19	5	31	8	50
			Second Largest	10	63	1	6	5	31

Courtesy Gann (Tokyo).

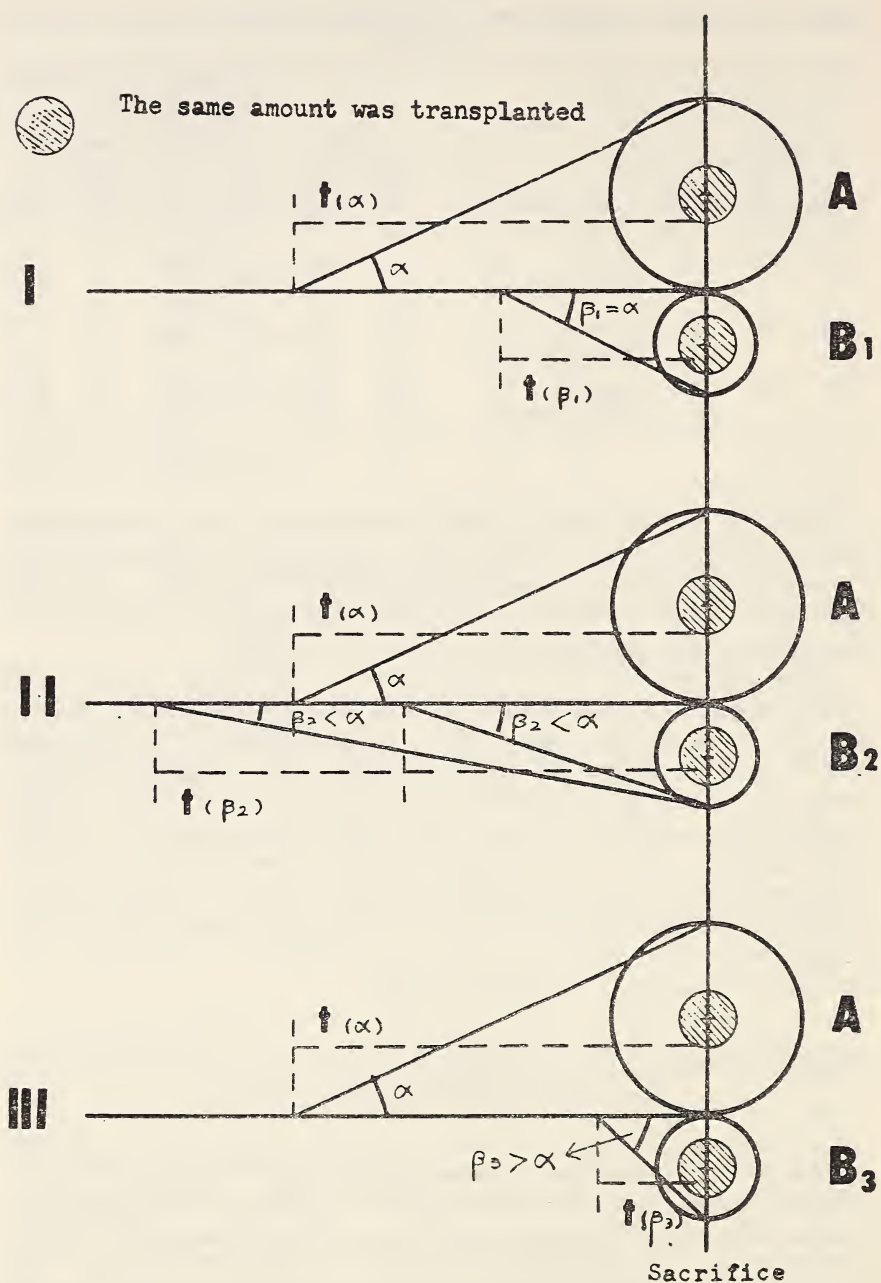
cell might be determined at the time of its conversion from a precancerous cell in which the effects of carcinogen are cumulative, and the total effect of carcinogen might depend on the dose given to the animals. All these speculations could be resolved completely if methods were discovered to determine the site and time that a cancer cell develops.

It has been stated that the transplantability of the largest tumor nodule was much greater than that of the second largest nodule. Text-figure 1 shows that the size of a tumor nodule is influenced by the proliferating power ( $\alpha$  and  $\beta$  1–3) and the period of proliferation. Type II shows that many cancer cells having different proliferating power might develop independently during the course of azo dye carcinogenesis, and that the most malignant cancer cell proliferates rapidly to become the largest tumor nodule which is the one that kills the host. In other cases, however, transplantation was successful only with the second largest tumor nodule which developed later than the largest one and did not reach the same size before the animal was killed (type III).

#### Correlation Between Transplantability and the Number of Experimental Days for Each Primary Tumor Animal

The time of death varied for each animal within a group of the same age and sex given the same kind and dose of carcinogen. Table 14 shows the relation between the number of days of the experiment and the incidence of animal deaths from primary tumor (14). In these cases, 0.05 percent 3'MeDAB was administered to 4 groups of thirty 6-week-old male Donryu rats for 3 to 6 months; this was followed by the basal diet. In the group fed for 5 months, the first animal died at day 170 and the last one at day 270. The results for the other groups were also similar.

The experimental period comprises the total time from the administration of the carcinogen until sacrifice. In this period, there were many complicating factors difficult to analyze, but in general, when transplan-



TEXT-FIGURE 1.—Schematic diagram of factors which influence the size of tumor nodules.

tation was done during the first part of the experimental period the transplantability rate was higher, with a greater convertibility into the ascites form (table 15).

TABLE 14.—Incidence of death from liver cancer in rats fed 3/MeDAB for 3 to 6 months

Group	Months of feed- ing	Num- ber of rats	Experimental months									
			1-6		1-7		1-8		1-9		1-10	
			Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
I	3	25	2	8	5	20	6	24	10	40	10	40
II	4	26	5	19	14	54	17	65	19	73	21	81
III	5	30	9	30	18	60	25	83	29	97	30	100
IV	6	30	9	30	20	67	28	93	30	100	—	—

TABLE 15.—Correlation between the time required for the development of liver cancer and transplantability

Months required		6	7	8	9	10	11	Total
Number of tumors transplanted intra-peritoneally		9	23	23	7	10	11	83
Positive ascites	Number	3	7	5	0	0	1	16
	Percent	33	30	22	0	0	9	19
Nodules, positive	Number	3	11	11	5	7	4	42
	Percent	33	52	48	71	70	36	51
Negative	Number	3	4	7	2	3	6	25
	Percent	33	17	30	29	30	55	30

### Correlation Between Transplantability and Histological Characteristics of the Primary Tumor Nodules

There have been many attempts (1, 16) to histologically classify azo dye-induced liver cancers. In our classification we have considered the relation of the parenchymal tumor cell arrangement, the fibrous stroma arrangement, and the positive acid-Schiff-(PAS) staining findings after the digestion of glycogen (13).

Type I, hepatomas: Cancer cells were arranged in cords and tubules and were sometimes parenchymatous with thin bands of fibrous tissue between the clusters. No PAS-staining substances could be found in the tissues. Two patterns were observed: In one, cancer clusters formed a cordlike or tubular arrangement surrounded by endothelial cells which were connected directly to the blood sinuses; these were called "typical" hepatoma (fig. 1). In the other, clusters of cancer cells formed a parenchymatous arrangement and were separated by thin bands of fibrous stroma with blood capillaries. In this pattern, blood sinuses usually failed to develop.

Type II, intermediate type: This type could be subdivided into two subtypes. (A) Cases with histological patterns resembling those of type I but including PAS-stained substances; there were few of these (fig. 2). (B) A pattern in which the arrangement of cancer cells was trabecular, tubular, follicular and, in some cases, papillotubular (fig. 3). In the latter, clusters of cancer cells exhibited a slight proliferation of intervening fibrous stroma and, in most of the cases, substances stained with PAS either within the cells or within the cavities of the clusters were included after glycogen digestion. In a few instances, squamous cell metaplasia of cancer cells (fig. 4) and formation of cartilage (fig. 5) or bone were found in the stromal tissues. In others, the stroma was abundant in cellular components and looked like embryonal connective tissues.

Type III, cholangiocarcinoma: The arrangement of tumor cells was similar to that in type II-B, but there was intensive proliferation of fibrous stroma in between the clusters of cells (fig. 6).

Type IV, carcinoma simplex: The cancer cells presented a polymor-

phous appearance, showed a variety of arrangements, and had thin connective tissues between the cells or small cell clusters (fig. 7).

On histological examination of induced liver cancers, a combination of the types mentioned was usually found. Sometimes, a single tumor nodule contained histologically different portions that were apparently separated from each other by fibrous stroma or normal liver tissues. In some, such lines of demarcation did not exist, and the different portions converged into each other. Such convergence was rare in types I and III.

#### Correlation Between the Occurrences of Various Histological Types and the Dose of Carcinogen Administered to Rats With Primary Tumors

The classification of the largest and the second largest tumor nodules developing in rats fed with 3' MeDAB for 3 to 6 months is shown in tables 16 and 17. The rate of occurrence is highest for type I and lowest for

TABLE 16.—Histological types of the largest and the second largest tumor nodules \*

Group	I		II		III		IV		
Months of feeding	3		4		5		6		
Number of rats	13		18		25		27		
Number of tumor nodules	A 13	B 0	A 18	B 8	A 25	B 12	A 27	B 16	
Histological types	I	5	—	6	3	6	6	8	11
	I + II	3	—	2	3	8	2	13	3
	I + II + IV	2	—	2	0	0	0	0	0
	I + IV	2	—	4	0	2	1	1	0
	II	0	—	1	1	4	2	2	1
	II + IV	1	—	2	0	2	1	1	1
	IV	0	—	1	1	3	0	1	0

\* A = largest nodule; B = second largest nodule.

TABLE 17.—Histological classification of primary tumor nodules from rats sacrificed in succeeding months

Experiment (months)			6	7	8	9	10	11
Number of rats			11	30	25	18	10	13
Histological types	I	Number Percent	27	74	71	12	31	33
			53	53	63	52	70	67
	II	Number Percent	15	50	24	8	6	7
			29	35	21	35	14	14
	III	Number Percent	1	2	3	1	1	1
			2	1	3	4	2	2
	IV	Number Percent	8	15	15	2	6	8
			16	11	13	9	14	16

type III. The incidence of type III was 3 percent in this experiment. But the incidence of liver cancers of type I was relatively higher for the longer experimental periods and that of types II and III was relatively lower. In addition, the rate of occurrence of type IV is high among the largest tumor nodules. However, definite correlation between the rate of occurrence of these types and the periods of azo dye-feeding was found.

### Correlation Between the Occurrence of Various Histological Types and the Incidence of Primary Tumor Ascites

Primary tumor ascites was found in about 30 percent of all the primary tumor animals. The largest and the second largest tumor nodules, in animals in which primary tumor ascites was found, were examined histologically and compared with those in animals in which primary tumor ascites was not found. The incidence of primary tumor ascites was generally higher when the tumors were of types II and IV (table 18).

TABLE 18.—Correlation between histological types of induced liver cancers and appearance of primary tumor ascites

Histological types	Number of rats	Primary tumor ascites			
		Positive		Negative	
		Number	Percent	Number	Percent
I	21	2	10	19	90
I + II	28	10	36	18	64
I + IV	11	4	36	7	64
I + II + IV	6	1	17	5	83
II	5	2	40	3	60
II + IV	4	0	0	4	100
IV	7	4	57	3	43

### Correlation Between the Transplantability of Induced Liver Tumors and the Histological Characteristics

Histological studies were made of the portions of tumor nodules which remained after transplantation. Tumor nodules of type IV seemed to have greater transplantability than those of type I, but the difference was slight (table 19). The convertibility into ascites form was not very different in tumor nodules of types I, II, and IV.

From the foregoing results, it may be said that the classifications based on hematoxylin and eosin-stained sections are not infallible indicators of transplantability and convertibility into ascites form.

TABLE 19.—Correlation between histological types of the primary tumor nodules and transplantability

Histological types	Results of transplantation					
	Largest nodule		Second largest nodule		Total	
		(%)		(%)		(%)
I	16/25	64	7/20	35	23/45	51
I + II	16/26	61	3/8	38	19/34	56
I + II + IV	4/4	100	0/0	0	4/4	100
I + IV	7/9	78	0/1	0	7/10	70
II	5/7	71	0/4	0	5/11	45
II + IV	3/6	50	2/2	100	5/8	63
IV	3/5	60	0/1	0	3/6	50
Total	54/82	67	12/36	33	66/118	56

Courtesy Gann (Tokyo).

#### “Direct” and “Indirect” Establishment and Conversion Into Ascites Hepatomas

Where conversion into ascites hepatoma was possible, two different types of proliferating cancer cells were observed in first transplant generation hosts. In the first, the inoculated tumor tissues developed in ascites form in hosts of the first transplant generation, and many proliferating cancer cells and/or clusters thereof could be detected in the tumor ascites. In the second, the inoculated tumor tissues produced no ascitic fluid in the first transplant generation, but cancer cells began to appear in the ascitic fluid of the host after the second transplant generation which could be used for serial transplantation of tumor ascites. In the first type, there was “direct” establishment and “direct” conversion (9). In the second type, there was “indirect” establishment and “indirect” conversion (9) (table 20). In 30 strains derived from primary tumor ascites all but 1 showed direct establishment. In 29 strains derived from tumor nodules, 16 showed direct conversion and the remaining 13 showed indirect conversion. Eight of the 13 strains were converted into ascites form before the fourth transplant generation, and the remaining 5 were converted between the eighth and fourteenth generations. In most indirect conversions, the number of tumor cells in the ascitic fluid before complete conversion was small and transplantation usually failed. However, if the number of cancer cells was sufficiently large, the conversion could be successful after fewer transplant generations.

The phenomenon of ascites conversion of the indirect form presents many problems. The following interpretations are based on histological observations. If two kinds of primary liver cancer are present in a simple tumor nodule, one kind may be capable of conversion into ascites form

TABLE 20.—Direct and indirect conversion into the ascites hepatoma

Types of conversion	Inoculum for the first transplant generation									
	Ascites					Nodules				
Direct	AH	108	130	66	63	AH	127	7974	601	49
		149	39	27	322		21	62	318	311
		364	414	423	173		41B	41C	131B	108B
		408	310	272	286		98B	84B	122B	65C
		344	131A	108A	98A					
		41A	109A	143A	150A					
		122A	371A	210A						
		225A								
Indirect	AH 55A(5)*					AH	137(3)*	602(2)	13(2)	
							99(9)	57B(2)	42B(2)	
							61B(3)	70B(4)	107B(8)	
							60C(9)	106B(9)	136B(3)	
							100B(14)			
Total	30					29				

\*The number in parentheses shows the generation in which tumor was converted into ascites form.

Courtesy Nagasaki Igakkai Zassi (Nagasaki).

while the other may not be. Moreover, the proportion of tumor tissue convertible into ascites form may be very small. The cancer cells possessing convertibility may be overshadowed by the other tumor tissue, and the animals may die before the development of tumor ascites. Through several transplant generations, the proportion of these convertible tumor cells may increase gradually; finally, tumor cells showing convertibility may overshadow other tumor tissues, and tumor ascites may develop after several generations. Cases in which such a course may be demonstrated are almost entirely limited to those in which apparently different tumor tissue patterns are involved. This was clearly demonstrated in the establishment of AH 99 (15), in which the greater part of each intraperitoneally induced tumor nodule up to the seventh transplant generation consisted of rhabdomyosarcoma and a few scattered tumor cells in a haphazard epithelial arrangement. In the eighth and ninth transplant generations, the hepatoma cells began to appear in the ascitic fluid, the myosarcomatous tissue tumor nodules gradually disappeared, and, thereafter, the ascites hepatoma AH 99 was established. This was a rarity in which the full course to ascites conversion for the indirect form could be followed. In other indirect conversions, a similar process might have occurred during ascites conversion, but there is no way to distinguish histologically. Furthermore, it is difficult to explain the cause of these phenomena and to know whether selection or adaptation was involved.

The procedure for the establishment of ascites hepatoma in rats may be summarized as follows:

- 1) Inbred animals should be used.
- 2) A large amount of strong carcinogen should be administered to the animals to induce primary liver cancers.
- 3) The primary tumor animals should be kept under observations for long periods

to accumulate tumor ascites. Large amounts of this ascites should be used for transplantation.

- 4) In transplantation with tumor nodules, inoculation should be carried out with the largest tumor nodule for each primary tumor animal.
- 5) If no tumor ascites develops in the first transplant generation, serial transplantations should be done for several generations to obtain indirect conversion into ascites form.

## GENERAL CHARACTERISTICS OF ASCITES HEPATOMA AND SPECIFIC CHARACTERISTICS OF EACH STRAIN

Fifty-four strains of ascites hepatoma established in the foregoing manner and 2 substrains are being carried in our laboratory for comparative studies. Twenty-nine strains were derived from hybrid Japanese rats, and 27 from Donryu rats.

The substrains AH 66F and 62F were derived from strains AH 66 and 62, respectively, during serial transplantation. They differed from the original strains, which are of island type, in that they proliferated mainly as single cells in ascitic fluid. With the exception of AH 601 and 602; AH 41A and 41B and 41C; AH 131A and 131B; AH 84A and 84B; and AH 122A and 122B, each of the 54 strains of ascites hepatoma originated in different primary tumor rats. The 11 strains listed were derived from primary tumor ascites, from the largest tumor nodule, or from the second largest tumor nodule obtained from 5 animals.

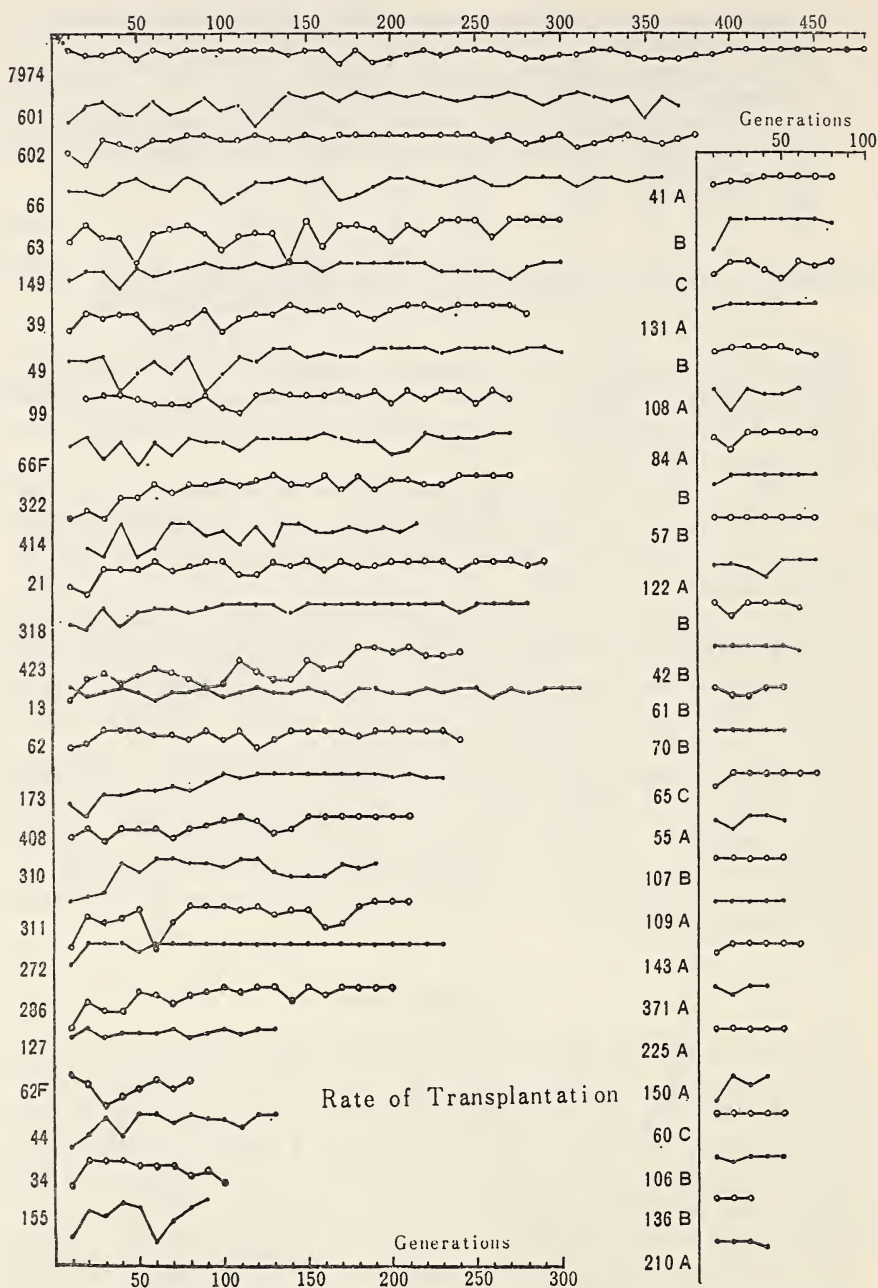
These 56 transplant strains of ascites hepatoma are being maintained by intraperitoneal transplantation of 0.1 to 0.3 ml (about  $2-3 \times 10^7$  cells) of tumor ascites once every 5 to 10 days; the transplantability, median survival time in days, and the pathological autopsy data are being recorded for each tumor animal. Some characteristics of ascites hepatoma are described as follows:

### Transplantability

The data for the first through the tenth generation and for the 10 most recent generations are described in tables 21 and 22. Transplantability in hybrids was 71 to 80 percent for 2 strains, 81 to 90 percent for 15, and 91 to 99 percent for 12 (table 23); in Donryu rats (table 24), it was 91 to 99 percent for 24 and 100 percent for 3 strains. In comparison of the average transplantability for the first to the tenth generation with that for the 10 most recent generations, the rate for the latter was often higher.

The transplantability in Donryu rats was much higher than in hybrids, and the rate in many of the ten most recent generations of Donryu rats with ascites hepatoma was 100 percent.

The average transplantability of each strain for every 10 generations to date is shown in text-figure 2. Thus, it is seen that the rate for each strain was not constant. Generally, the rate increased, as seen for strains AH 286, 423, 322, 49, and 39. Yet, the transplantability remained



TEXT-FIGURE 2.—Average transplantability of each strain for every 10 generations.

high throughout for strains AH 7974 and 13. In Donryu rats, the transplantability was generally higher from the beginning and in some was 100 percent throughout.

TABLE 21.—Generations, transplantability, and survival time in days for the ascites hepatoma-bearing animals (hybrid)

Strains	Estab- lished (year)	Genera- tion	Takes (%)	Survival (days)	First to tenth generation		Ten most recent generations	
					Takes (%)	Survival (days)	Takes (%)	Survival (days)
1	AH 130	1951	520	97	12	94	12	97
2	7974	1952	480	97	12	100	15	100
3	601	"	371	87	15	65	25	86
4	602	"	382	94	19	79	21	100
5	66	1954	360	93	11	85	16	100
6	63	1955	300	85	13	75	35	100
7	149	"	301	93	11	81	19	100
8	39	"	281	90	14	68	14	90
9	49	"	301	88	13	84	17	97
10	99	"	272	92	10	88	16	90
11	66F	1956	271	89	10	85	9	100
12	322	1957	172	87	11	50	19	100
13	414	"	221	80	16	100	31	96
14	21	"	293	93	8	70	10	100
15	318	"	281	95	8	77	12	100
16	423	"	241	75	14	35	30	96
17	13	"	302	96	7	100	7	100
18	62	"	241	95	11	83	20	92
19	173	1958	231	90	9	64	20	97
20	408	"	211	90	11	75	17	100
21	310	"	194	85	13	53	24	93
22	311	"	211	83	11	44	17	100
23	272	"	231	99	6	77	25	100
24	286	"	201	89	10	52	13	100
25	127	"	131	95	17	90	18	100
26	62F	1959	80	82	18	95	13	91
27	44	"	130	89	9	61	13	100
28	34	1960	101	85	15	65	25	70
29	155	"	90	81	15	55	15	100

Transplantability of a given transplantable tumor was determined from the percentage of animals that died as a result of the proliferation of the inoculated tumor cells, *i.e.*, the adaptability of the tumor cell against the host animals inoculated determines the transplantation rate. If the constitution of the animal is entirely compatible with that of the primary tumor animal, the inoculated tumor cells can always grow in the new host and the transplantability should be 100 percent. Even though the constitution of the animals is not entirely compatible, the rate may still be the same, provided that the compatibility range is not too great.

The transplantability in Donryu rats is much higher than in hybrid rats because the constitution of the former is relatively more homogeneous. Nevertheless, hybrid animals are often used, and it has been found that there is an increase in transplantability after serial transplantation for a number of generations. During extended periods of serial transplantation, cancer cells may undergo changes and become compatible *vis-a-vis* the animal. Another interpretation is also possible. A mixture of transplantable cancer cells with different degrees of compatibility may be taken from the primary tumor nodule. During early transplant generations, these cancer cells showing considerable compatibility may grow, but the animals may be cured before these cells proliferate sufficiently.

TABLE 22.—Generations, transplantability, and survival time in days for the ascites hepatoma-bearing animals (Donryu)

	Strains	Established (year)	Generation	Takes (%)	Survival (days)	First to tenth generation		Ten most recent generations	
						Takes (%)	Survival (days)	Takes (%)	Survival (days)
1	AH 41A	1960	111	98	12	89	12	97	14
2	41B	"	111	97	10	67	12	100	8
3	41C	"	111	96	10	85	12	100	11
4	131A	"	111	100	10	95	14	100	10
5	131B	"	111	93	13	95	14	100	12
6	108A	"	91	93	18	100	20	92	17
7	84A	"	111	98	10	95	13	100	9
8	84B	"	101	99	12	90	16	100	11
9	57B	"	111	99	12	100	10	100	11
10	122A	1961	91	97	12	95	11	100	15
11	122B	"	91	98	14	100	13	100	14
12	42B	"	81	97	17	100	15	97	18
13	61B	"	91	96	11	100	9	100	10
14	70B	"	81	98	16	100	16	100	16
15	65C	"	101	99	10	84	13	100	10
16	55A	"	81	97	15	95	14	100	16
17	107B	"	81	100	9	100	12	100	9
18	109A	"	81	98	13	100	14	100	12
19	143A	"	91	98	10	90	13	97	11
20	371A	"	91	97	14	100	16	96	14
21	225A	"	81	100	11	100	10	100	14
22	150A	"	81	93	13	65	19	100	12
23	60C	"	91	99	12	100	11	100	13
24	106B	"	81	99	11	100	14	100	10
25	136B	"	61	99	18	100	18	96	20
26	210A	"	81	98	9	100	9	97	9
27	100B	1962	31	94	18	89	25	100	16

If the tumor cells become dominant, however, the transplantability may gradually increase.

#### Median Survival Time of Animals in Days

The results obtained are shown in tables 25 and 26. The number of days at which 50 percent of the animals with positive transplants died was indicated as the time. The shortest was 6 days (AH 272); the longest was 19 days (AH 602). In most rats, the median survival time was 11 to 15 days, and there is no difference between the Donryu and the hybrid rats. These averages were obtained with intraperitoneal inoculations of 2 to  $3 \times 10^7$  cells. With smaller numbers of tumor cells, the median survival time increased.

In text-figure 3, the median survival time in days for every 10 generations is shown for each strain. There was a general tendency toward a slight decrease in survival time with strains AH 601, 149, and 272. Strains AH 7974 and 127, however, showed constant survival time throughout, and strains AH 602, 423, and 286 showed sudden changes at the 290th, 60th, and 80th transplant generation, respectively.

The median survival time might indicate the degree of virulence of the proliferating cancer cells. The results obtained show that, in each case of azo dye-induced liver cancers, cells derived from common ancestors have varying degrees of virulence.

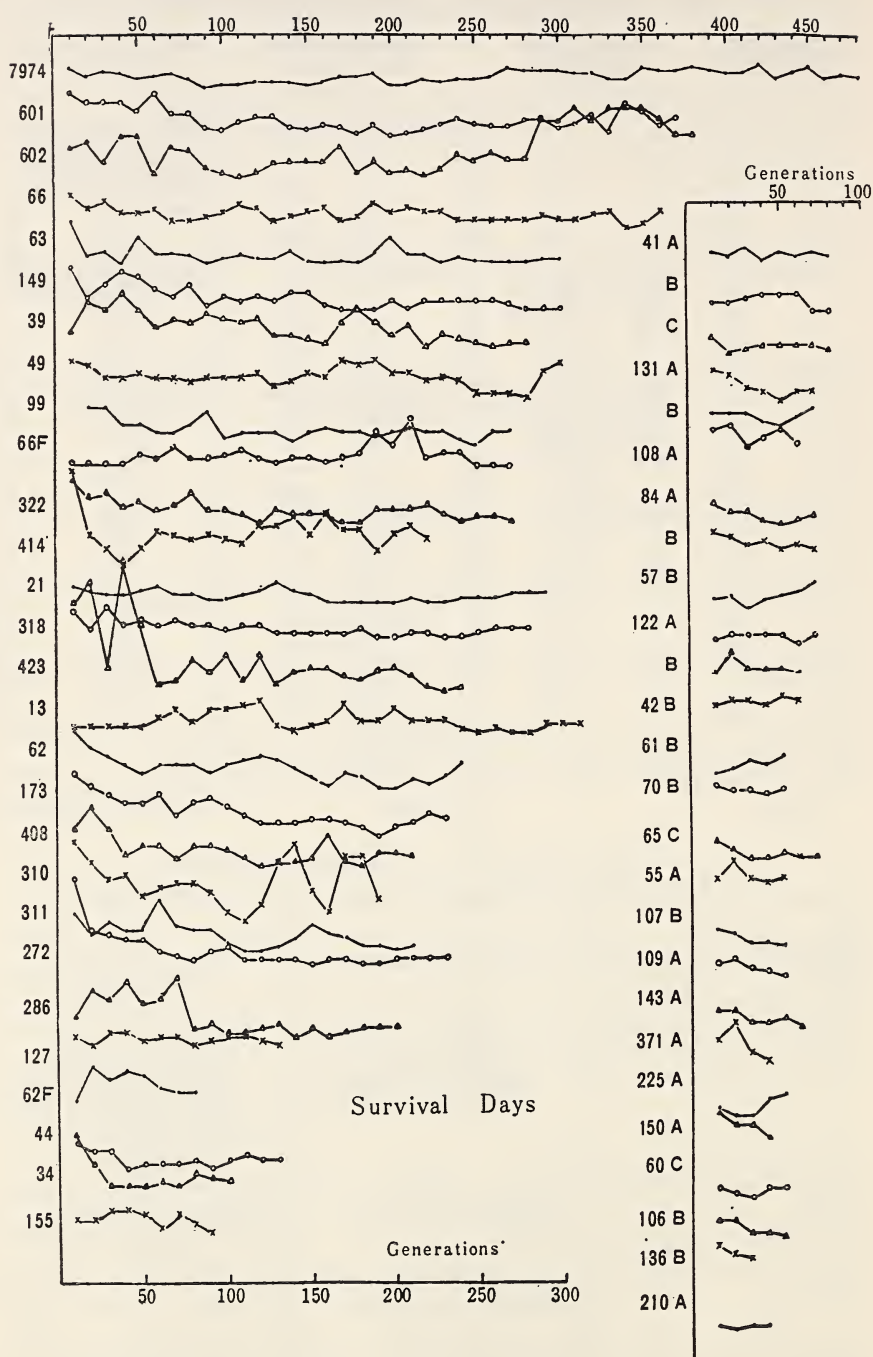
TABLE 23.—Transplantation rate for the ascites hepatoma (hybrid)

Transplant-ability (%)	First to latest generations	First to tenth generations	Ten most recent generations
100		AH 7974, 414, 13 3	AH 7974, 602, 66, 63, 149, 66F, 322, 21, 318, 13, 408, 311, 272, 286, 127, 44, 155 17
91-99	AH 130, 7974, 602, 66, 149, 99, 21, 318, 13, 62, 272, 127 12	AH 130, 62F 2	AH 130, 49, 414, 423, 62, 173, 310, 621 8
81-90	AH 601, 63, 49, 66F, 39, 322, 173, 408, 310, 311, 286, 62F, 44, 34, 155 15	AH 66, 149, 49, 99, 66F, 62, 127 7	AH 601, 39, 99 3
71-80	AH 414, 423 2	AH 602, 63, 318, 408, 272 5	
61-70		AH 601, 39, 21, 173, 44, 34 6	AH 34 1
51-60		AH 310, 286, 155 3	
41-50		AH 322, 311 2	
31-40		AH 423 1	

TABLE 24.—Transplantation rate for the ascites hepatoma (Donryu)

Transplant-ability (%)	First to latest generations	First to tenth generations	Ten most recent generations
100	AH 107B, 225A, 131A 3	AH 108A, 57B, 122B, 42B, 61B, 70B, 107B, 109A, 371A, 225A, 60C, 106B, 136B, 210A 14	AH 41B, 41C, 131A, 131B, 84A, 84B, 57B, 122A, 61B, 70B, 65C, 107B, 109A, 122B, 55A, 225A, 60C, 106B, 150A, 100B, 20
91-99	24	AH 131A, 131B, 84A, 122A, 55A 5	AH 41A, 108A, 143A, 42B, 371A, 136B, 210A 7
81-90		AH 41A, 41C, 84B, 65C, 143A, 100B 6	
61-70		AH 41B, 150A 2	

Courtesy Nagasaki Igakkai Zassi (Nagasaki).



TEXT-FIGURE 3.—Median survival time in days for every 10 generations for each strain.

TABLE 25.—Survival time for the ascites hepatoma-bearing animals (hybrid)

Survival time (days)	First to latest generations	First to tenth generations	Ten most recent generations
5-10	AH 21, 318, 13, 173, 272, 44, 99, 66F, 286 9	AH 66F, 13 2	AH 149, 66F, 322, 21, 423, 318, 13, 173, 311, 408, 272, 44, 130, 99, 310, 286 16
11-15	130, 7974, 601, 66, 63, 149, 39, 49, 322, 423, 62, 408, 310, 311, 34, 155 16	130, 7974, 39, 318, 286, 62F, 44, 155 8	74, 66, 63, 39, 414, 62, 62F, 34, 155 9
16-20	602, 414, 127, 62F 4	66, 149, 49, 99, 322, 62, 173, 408, 311, 127 10	601, 49, 127 3
21-25		601, 602, 310, 272, 34 5	602 1
26-30		423 1	
31-25		63, 414 2	

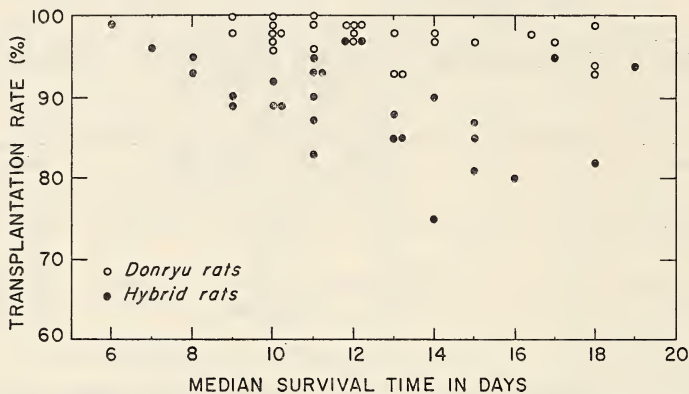
TABLE 26.—Survival time for the ascites hepatoma-bearing animals (Donryu)

Survival time (days)	First to latest generations	First to tenth generations	Ten most recent generations
5-10	AH 41B, 41C, 131A, 84A, 65C, 107B, 143A, 225A, 210A 9	AH 57B, 61B, 225A, 210A 4	AH 41B, 41C, 131A, 84A, 65C, 107B, 143A, 106B, 210A 9
11-15	41A, 131B, 84B, 57B, 122A, 122B, 61B, 70B, 55A, 109A, 371A, 150A, 60C, 106B 14	41A, 41B, 41C, 131A, 131B, 84A, 122A, 122B, 42B, 65C, 55A, 109B, 371A, 225A, 150A, 60C, 136B 17	41A, 131B, 84B, 57B, 122A, 122B, 61B, 70B, 55A, 109A, 371A, 225A, 150A, 60C, 136B 15
16-20	108A, 42B, 136B 3	108A, 84B, 70B, 371A, 150A, 136B 6	108A, 42B 2
21-25	100B 1	100B 1	100B 1

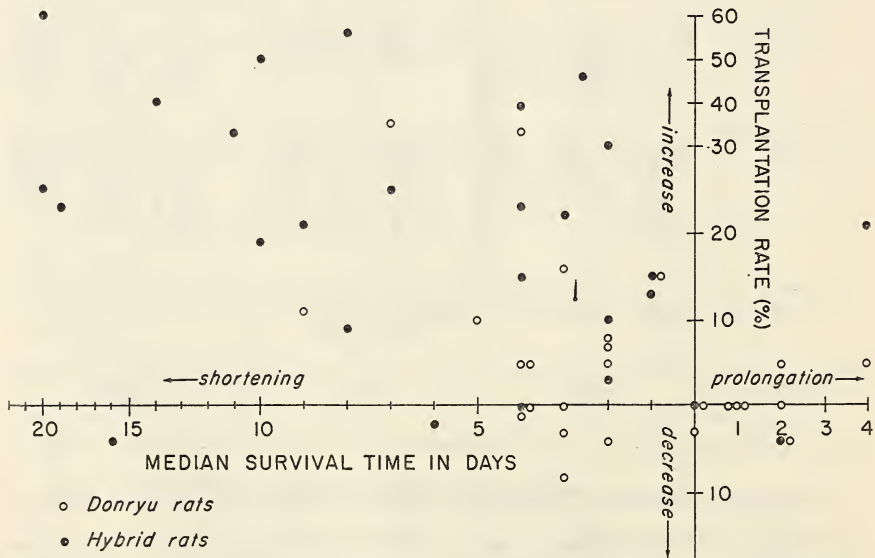
## Correlation Between Transplantability and Survival Time in Days

Text-figure 4 shows that in most of the strains of hybrid rats the transplantability increased as the median survival time decreased. In Donryu strains, in which the transplantability was 95 to 100 percent, there was no correlation.

In text-figure 5 the difference in degree of transplantability for each strain, averaged over the first to tenth generations as compared with that for the 10 most recent generations, is shown along the vertical axis (above the line indicates increased transplantability, below indicates decreased); the difference in the median survival times for each strain is shown along the horizontal axis (to the left indicates shortening, the right indicates prolongation). In most instances, there was an increase in transplantability accompanied by a shortening of survival time; in a few instances, there was an increase with prolongation, a decrease with shortening, a decrease with prolongation, or no relative change.



TEXT-FIGURE 4.—Correlation between transplantation rate and median survival time in days for animals bearing each ascites hepatoma strain.



TEXT-FIGURE 5.—Transplantation rate and survival time. Average of the first to tenth generations compared with the 10 most recent generations for each strain of ascites hepatoma.

### Characteristics of Tumor Ascites

In the normal rat, a small amount of transparent or slightly opalescent ascitic fluid is found. On the average, it contains  $3$  to  $5 \times 10^7$  per ml of white blood cells, mostly lymphocytes and monocytes. The total volume of fluid is about  $0.1$  to  $0.3$  ml.

After inoculation with ascites hepatoma cells, the ascitic fluid gradually becomes opaque because of an increase in tumor cells; there is also hemorrhage in most animals. Tumor ascites without hemorrhage is thick and white; that with intensive hemorrhage has the appearance of diluted whole blood. The concentration of tumor cells in the fluid gradually increases and reaches  $1$  to  $2 \times 10^8$  ml  $4$  to  $7$  days after inoculation with  $1$  to  $3 \times 10^7$  tumor cells; the volume totals about  $0.5$  to  $2.5$  ml. With some strains, the majority of animals died under such conditions; with other strains, the animals survived and produced a large amount of tumor ascites, whose density gradually became diluted with a decrease of tumor cells. With invasion and obstruction of the common bile duct by tumor and resulting jaundice, there is a marked decrease in tumor cells in ascitic fluid.

The number of tumor cells in  $1$  ml and the degree of hemorrhage in the ascitic fluid vary with the number of tumor cells inoculated and the number of days after inoculation; each ascites hepatoma strain exhibits different characteristics. In some strains, for example, the ascites exhibits intensive hemorrhage several days after inoculation; in other strains, the tumor ascites exhibits no hemorrhagic tendency and remains white for many days. The density of ascites varies for different strains. In some animals, the ascites sometimes became so thick that it was impossible to draw it up with a glass capillary; in others, the density was relatively low throughout the lifespan of the animal. The amount of tumor ascites was gradually larger in the latter than in the former.

The volume percentages of tumor cells, erythrocytes, and fluid components for 4-day-old tumor ascites for each strain are shown in table 27. The volume of tumor cells was between  $12$  to  $54$  percent, erythrocytes were  $0$  to  $13$  percent, and the remaining portion was fluid. In animals with extensive hemorrhage,  $4$  to  $5$  mg per  $100$  ml of hemoglobin was detected; this is about one third the amount found in the peripheral blood of normal rats.

In previous examinations, no apparent correlation was found between the degree of hemorrhage and the median survival time in days or the transplantability.

### Infiltrating Tumor Nodules Into Peritoneal Tissue

In rat ascites hepatoma, infiltration of tumor nodules into the peritoneal tissue was usually found in addition to the accumulation of tumor ascites previously mentioned. Generally, the infiltration was quite variable and did not always appear to be related to medium survival time (table 28). When small numbers of tumor cells were transplanted, the lifespan was prolonged and the degree of infiltration increased.

TABLE 27.—Structure of tumor ascites 4 days after inoculation

Strain	Cell components		Fluid (%)	Survival time in days
	Tumor cells (%)	Erythrocytes (%)		
AH 13	38	8	54	6
42B	54	6	40	8
41C	39	11	50	9
131A	21	6	73	9
131B	22	±	78	15
108A	38	6	56	17
84A	18	2	80	10
84B	49	11	40	12
122B	31	9	60	13
42B	44	10	46	16
61B	53	12	35	13
70B	47	11	42	15
60C	50	2	48	11
106B	21	7	72	10
210A	12	8	80	9
371A	31	13	56	11
109A	12	±	88	11
55A	41	9	50	14

TABLE 28.—Extent of infiltration into intraperitoneal tissue

Survival time in days	High	Medium	Low
5-10	AH 57B, 60C	AH 99, 173, 41C, 61B, 65C, 225A, 210A	AH 66F, 21, 318, 13, 272, 44
11-15	130, 7974, 39, 49, 84A	63, 149, 322, 408, 311, 286, 41A, 41B, 131A, 131B, 122A, 42B, 70B, 55A, 107B, 143A	601, 66, 423, 62, 310, 34
16-	414, 127, 150A, 106B, 136B	62F	602, 155, 108A

Infiltrating nodules were usually found in the omentum major, sometimes in the omentum minor, pancreas, ligament gastrolieal, genitourinary tissues, and diaphragm, and, occasionally, in the parietal peritoneum. They sometimes occurred in the subcutaneous tissues, usually at the site of the inoculation puncture.

In most of the animals, the infiltrating nodules were soft and rich in blood capillaries. In some strains, such as AH 130, 414, 21, and 371A, multiple nodules resembling bunches of grapes were found in the omentum. In other rats, hard nodules formed in the omentum and were accompanied by abundant fibrous connective tissue.

Most of the infiltrating nodules exhibited a histological picture of parenchymatous hepatoma; a few were typical hepatomas (AH 55A, 107A) or intermediate types (AH 51B, 106B, and 131B). In general, the former increased in number with successive transplant generations.

### Cancer Cells in the Peripheral Circulating Blood and Incidence of Remote Metastases

Besides accumulation of tumor ascites and formation of infiltrating nodules into the peritoneal tissue, metastases to the lungs and mediastinal lymph nodes and accumulation of tumorous pleural fluid sometimes occurred. This was particularly pronounced in animals exceeding the median survival time, sometimes as a result of cancer chemotherapy. Usually, the animals died before such large tumor nodules could be detected macroscopically.

These findings were supported by the frequent detection of tumor cells in the circulating blood. The detection of cancer cells was studied by the transplantation of the heart blood of the tumor-bearing animals into other animals and by the examination of smears with sediment of heart blood (8). The results of such bioassay were comparable to those for transplantation with small numbers of tumor cells and were more sensitive than the smear test. The results obtained with 10 ascites hepatoma strains indicate that in strains AH 13 and 66F tumor cells can be detected in the circulating blood within 24 hours after inoculation (table 29). This observation is quite important for the early detection of metastases when surgery and X-ray therapy are usually unsatisfactory for cancer therapy.

TABLE 29.—Detection of tumor cells in heart blood of tumor-bearing animals

Strain	Days after intra-peritoneal inoculation		Strain	Days after intra-peritoneal inoculation	
	Transplantation test	Smear test		Transplantation test	Smear test
AH 13	1	3	AH 63	7	13
66F	1	5	62	7	
130	3	6	322	7	
21	3	7	99	7-10	10
7974	4	8	602	7-10	
149	4	9	601	7-10	
49	4	10	414	7-10	
39	4	15	423	10-15	
66	4		364	10-15	9
318	7	7			

The cancer cells found in the heart blood usually were single or double, and larger congregations usually were rare. This would seem to suggest that the smaller the clusters of cancer cells in the ascitic fluid the more frequent was the appearance of tumor cells in the heart blood. However, as shown in table 30, this is not true. The table gives the names of the strains for which, by the usual methods of inoculation, remote metastases to the mediastinal lymph nodes were found on autopsy and presents the findings in the ascites. With strains AH 49 and 322, though the hepatoma islands were normally composed of more than 20 cancer cells, the incidence of remote metastases was high; in some strains which formed smaller

TABLE 30.—Size of hepatoma islands in strains in which vigorous metastasis to mediastinal lymph nodes occurs

Strain	Free cell (%)	2- to 4-celled island (%)	Island bigger than 5-celled island (%)
AH 13	97	3	0
66F	100	0	0
414	97	3	0.4
130	94	4	2
66	40	26	34
62	23	10	67
7974	18	50	32
173	16	15	69
49	9	14	77
44	7	21	72
322	6	12	82
149	5	14	80
62F	99	1	0.5
127	20	19	62

islands, remote metastases were seldom found. This shows that the incidence of metastases has no relation to the size of the hepatoma islands, *i.e.*, of the capacity of the cancer cells to adhere together.

The results presented in table 31 seem to indicate that there is no distinct correlation between incidence of remote metastases and median survival time in days for each strain. Generally, however, animals that exceeded the median survival period experienced more metastases for each strain of ascites hepatoma.

TABLE 31.—Incidence of metastasis to mediastinal lymph nodes of tumor-bearing animals

Survival time in days	High	Medium	Low
5-10	AH 131A	AH 173, 318, 21, 13, 210A, 225A	AH 44, 272
11-15	322, 49, 39, 130, 7974, 41A, 41B, 84B, 122B, 109A	408, 66, 66F, 63, 99, 286, 149, 311, 41B, 41C, 131B, 42B, 122A, 57B, 84A, 61B, 70B, 65C, 55A, 107B, 60C, 106B, 143A	34, 62, 423, 310, 601
16-20	127, 155	602, 136B, 371A, 150A	108A, 62F, 414

### Microscopic Character of Tumor Ascites

Microscopically, the tumor ascites is composed of tumor cells, blood cells, and reactive cells of the fluid, such as leukocytes, lymphocytes, and monocytes.

The usual staining methods employed for the examination of tumor ascites are Wright-Giemsa staining and Papanicolaou staining of smear preparations. With strains composed of free tumor cells and/or small clusters of less than about five cells, the Wright-Giemsa method was the best. However, this method is not suitable for strains forming larger clusters, since the entire cluster stains a uniform dark blue, and it is difficult to differentiate the nuclei from the cytoplasm. In such case, Papanicolaou staining is suitable. Moreover, if it is unnecessary to keep the specimens for a long period, acetic-gentian violet staining, orcein staining, or acetic dahlia staining can also be used. In serial transplantation to maintain tumor strains, acetic-gentian violet method was usually employed.

Lymphocytes, monocytes, pseudoeosinocytes, and small numbers of leukocytes are observed in the ascitic fluid of normal rats. After inoculation with tumor ascites, the ratio of tumor cells to reactive cells changed gradually; the constitution of the reactive cells also changed within a few hours.

With  $2$  to  $3 \times 10^7$  tumor cells, the proliferating tumor cells were observed in a pure culture 72 to 96 hours after inoculation, and only a few reactive cells were found in the tumor ascites. This is the most useful stage for cytological examination of the tumor cells, since neither hemorrhage nor degeneration exists to promote the formation of reactive cells.

A specific characteristic of most ascites hepatomas is that the cancer cells in ascitic fluid grow in clusters. This is not true of many other ascites tumors. The clusters of cells—"hepatoma islands"—vary in size. The smallest cluster was 2 cells and was called "double." Three-celled (triple), 4-celled, and 5-celled islands were considered as small clusters. Larger ones consisted of more than 100 tumor cells; some of these islands appeared macroscopically as a white mass. Even the largest islands were composed of cancer cells only, and no cell elements such as connective tissues and blood capillaries could be found.

The average size of these islands varied for each strain. In the majority of cases in which small islands were found, the clusters remained small for many transplant generations. However, if the islands were large—more than about 100 tumor cells—in the earlier transplant generations, they became smaller with successive transplantation and after a certain period were consistently of moderate size (about 10–30 tumor cells). In many cases, this was accompanied by an increase in transplantability and a decrease in the median survival time.

Examination of paraffin sections of hepatoma islands revealed that the islands were round or oval, but some were trabecular or tree-shaped. Most of the islands were parenchymatous, but a few (AH 602) were adenomatous with a single or double layer of cells. Islands composed of 5 to 10 layers of cells usually exhibited degeneration or necrosis in the central part of the island. Apparently, this phenomenon resulted from inadequate nutrition of the cells at the center, which seems to be the principal reason for the limited size of hepatoma islands.

TABLE 32.—Percentage of various sized hepatoma islands and single cells in 4-day-old tumor-bearing animals of each strain (hybrid)

Strain	Single	Double	Triple	4 Cells	5 to 10 Cells	Larger than 10 cells
AH 66F	100.0	0	0	0	0	0
62F	98.6	1.2	0	0.5	0.5	0
13	97.4	2.5	0.1	0	0	0
414	96.8	2.2	0.4	0.3	0.3	0.1
39	96.1	3.4	0.3	0.1	0.1	0.1
130	94.1	3.1	0.4	0.1	1.3	1.0
21	90.1	7.7	0.9	0.5	0.6	0.4
423	82.4	5.0	3.4	3.1	4.9	1.8
272	72.5	9.2	3.3	1.9	6.1	7.0
66	39.8	13.6	7.9	4.7	17.5	16.7
63	29.8	25.7	11.7	8.0	17.1	7.6
62	22.9	7.4	3.6	1.3	15.1	51.9
34	21.8	15.5	8.8	5.9	20.5	26.3
127	20.0	11.7	3.9	2.8	25.7	25.8
7974	17.7	28.6	13.0	7.6	16.1	16.9
173	16.3	8.3	3.8	2.8	7.0	61.9
311	15.9	15.9	7.3	3.7	19.8	37.3
310	13.2	13.9	5.4	2.1	12.7	52.5
408	11.4	13.9	4.4	2.5	10.0	58.2
49	8.8	8.4	3.6	1.9	16.9	60.4
318	8.5	13.4	4.5	2.5	12.3	58.9
44	7.3	0.9	6.0	4.7	37.9	34.2
601	6.5	8.1	4.5	3.4	18.8	58.9
602	6.5	3.1	1.2	0.9	12.3	75.9
322	6.1	6.2	3.5	1.7	11.8	70.1
149	5.3	7.5	3.5	3.5	32.1	48.2
99	3.6	5.8	1.5	1.2	13.1	75.6
286	1.8	1.8	0.6	0.6	3.6	93.1

The foregoing results show that each strain has characteristics which differentiate it from other strains. Strains AH 13, 62F, and 66F usually develop single cells, whereas strain AH 49 consists largely of hepatoma islands of more than 50 cells. Double and triple cells are common in strain AH 39. Since these characteristics are usually specific for various strains, it is sometimes possible to identify the strain merely after examination of the tumor ascites. Tables 32 and 33 show various sized hepatoma islands for each strain as found in 4-day-old tumor-bearing animals. In some strains, single cells were found about 100 percent of the time but in other strains constituted less than 3 percent (figs. 8 and 9). Thus, it was possible to distinguish strains of ascites hepatomas by examination of the ascites, but it was impossible to make such a distinction by histological means in the case of a nonconvertible nodular type of hepatoma.

#### Establishment of Substrains of Ascites Hepatoma

Except for strains AH 41A, B, C; AH 131A, B; AH 84A, B; AH 122A, B; and AH 601 and 602, the characteristics of ascites hepatomas described are based on ascites hepatomas derived from different primary tumor animals. The character of the ascites, the transplantability, and the median survival time varied somewhat over long periods of

TABLE 33.—Percentage of various sized hepatoma islands and single cells in 4-day-old tumor-bearing animals of each strain (Donryu)

Strains	Single	Double	Triple	4 Cells	5 to 10 Cells	Larger than 10 cells
AH 210A	96.8	1.4	0.6	0.3	0.7	0.3
41C	94.3	2.1	0.8	0.6	1.5	0.6
225A	92.8	6.2	0.7	0.3	0.1	0
131B	92.1	5.6	1.0	0.6	0.7	0.1
109A	79.3	4.8	1.7	1.5	6.0	6.7
131A	33.0	21.8	10.5	8.9	20.1	5.6
41B	26.0	15.7	9.6	9.3	31.2	8.4
41A	19.9	10.8	9.6	8.4	34.6	16.7
84A	14.8	7.9	3.2	1.6	6.8	65.6
150A	14.0	11.5	3.6	2.1	17.7	51.1
61B	10.3	39.0	11.2	5.6	16.4	17.4
122B	8.8	6.3	2.9	1.8	9.8	70.4
60C	7.1	8.3	4.4	3.3	19.1	57.9
42B	6.9	4.3	1.7	1.3	15.3	70.5
65C	5.6	6.6	2.7	1.9	14.5	68.6
107B	5.2	8.1	2.4	1.6	9.9	72.8
122A	4.7	4.8	2.4	2.5	24.1	61.5
84B	4.1	3.8	2.1	1.2	10.1	78.7
143A	3.7	1.9	0.6	0.4	5.7	87.8
136B	2.2	1.5	1.2	0.7	10.8	83.7
70B	2.2	1.6	0.7	0.9	5.3	89.2
55A	1.4	1.4	1.2	0.8	10.5	84.8
106B	1.0	1.0	0.5	0.5	8.1	88.9
371A	1.0	0.8	0.2	0.3	2.4	95.3
57B	0.8	1.2	1.0	0.9	6.1	90.1
108A	0.6	0.5	0.5	0.5	9.1	88.8

transplantation. However, this need not suggest any essential changes in the cancer cells. For example, the phenomenon whereby hepatoma islands became smaller through serial transplantation may have resulted from adaptation or selection which could have occurred over a long period of ascites growth. However the question remains whether we have adequate methods to distinguish various tumor strains, including those that actually developed from other strains.

Strains AH 66F from AH 66 and strain 62F from AH 62 seemed to result from essential changes in the cancer cells. Both substrains were converted from the island type to the free cell type of ascites hepatoma in a certain transplant generation and, thereafter, remained in the free cell state. Strain AH 66F was detected in animals in which the usual serial transplantation technique was employed, and during the following 3 transplant generations almost all the cancer cells observed were free tumor cells. Strain AH 62F was found in 1 animal in which tumor ascites of AH 62 was transplanted after 111 days of storage at  $-80^{\circ}\text{C}$ . Both of the control strains and both substrains have maintained their original character to date (table 34). Most remarkable is the difference in sensitivity to Nitromin ( $\text{HN}_2$  N-oxide). Thus, the sensitivity of the control strain AH 66 was about 50 times greater than that of substrain AH 66F. The reason for the establishment of the substrain is very difficult to ascertain, but it is possible that mutation of the cancer cells of strain AH 66 might have occurred.

TABLE 34.—Comparison of AH 66, AH 62, and substrains thereof

	AH 66	AH 66F	AH 62	AH 62F
Survival time in days	13	10	11	18
Transplantability (%)	93	89	95	82
Composition of tumor ascites (%)				
Fluid	84	50	74	64
Erythrocytes	0-1	0-1	2	3
Tumor cells	16	50	24	33
Single cell	40	100	23	99
Sensitivity to Nitromin (MED:mg/kg)	50	1	20	20

## SUMMARY

Results of a series of experiments on the establishment of ascites hepatomas in rats and the general characteristics of the strains established are described. Results of other experiments such as chromosomal studies, studies on cancer chemotherapy, and the drug resistance of cancer cells from these ascites hepatoma strains are described elsewhere in this Monograph.

The author believes that ascites hepatomas are very valuable in the field of cancer research and hopes that the use of these rat ascites hepatomas will help to solve many problems.

## REFERENCES

- (1) EDWARDS, J. E., and WHITE, J.: Pathologic changes, with special reference to pigmentation and classification of hepatic tumors in rats fed *p*-dimethylaminoazobenzene (butter yellow). *J Nat Cancer Inst* 2: 157-183, 1941.
- (2) GOLDIE, H., and FELIX, M. D.: Growth characteristics of free tumor cells transferred serially in the peritoneal fluid of the mouse. *Cancer Res* 7: 430-437, 1954.
- (3) IRAKO, Y.: Studies on the effect of X-irradiation upon the ascites tumor cells. II. Different sensitivities of various transplant strains of the ascites hepatoma to X-irradiation. *Gann* 49 (Suppl): 319-320, 1958.
- (4) ISAKA, H.: Transformation of the OAT-hepatoma into ascites form. *Gann* 44: 174-176, 1953.
- (5) ISAKA, H., NAKAMURA, K., and ODASHIMA, S.: Studies on the ascites hepatoma. V. Transplantation of ascites hepatoma with a single cell. *Gann* 45: 434-436, 1954.
- (6) ———: Studies on the ascites hepatoma. VII. On the chromosome number of the ascites hepatoma cells derived from a single cell. *Gann* 46: 194-196, 1955.
- (7) KAZIWARA, K., ISAKA, H., NAKAMURA, K., ARUJI, R., KAISE, A., ODASHIMA, S., and SATOH, H.: Studies on the ascites hepatoma. II. On the transplantation rate of the ascites hepatoma intraperitoneally transplanted. *Gann* 44: 307-309, 1953.
- (8) KURATA, T.: Hematogeneous dissemination of tumor cells. Studies with the ascites hepatoma of the rat. *Trans Soc Path Jap* 48: 1329-1337, 1959.

- (9) KLEIN, G., and KLEIN, E.: Conversion of solid neoplasms into ascites tumors. *Ann NY Acad Sci* 63: 640-661, 1956.
- (10) NAKAMURA, K.: Studies on the ascites hepatoma. VIII. Difference of chromosome numbers among tumor cells constituting the same "island" of the ascites hepatoma. *Gann* 46: 196-199, 1955.
- (11) NAKAMURA, K., ODAHIMA, S., ISAKA, H., and KURATA, T.: Studies on the ascites hepatoma. X. Further studies on the chromosome number. *Gann* 47: 502-504, 1956.
- (12) ODASHIMA, S.: Studies on the ascites hepatoma. XXI. Ascitic conversion of the liver cancers developed in rat fed with 3'Me-4-DAB. *Proc Jap Cancer Ass, The 20th General Meeting*. *Gann* 52 (Suppl): 91, 1961.
- (13) ———: Development of liver cancers in the rat by 20-methylcholanthrene painting following initial 4-dimethylaminoazobenzene feeding. *Gann* 50: 321-345, 1959.
- (14) ———: Comparative studies on the transplantability of liver cancers induced in rats fed with 3'-methyl-4-dimethylaminoazobenzene for 3 to 6 months. *Gann* 53: 325-348, 1962.
- (15) ODASHIMA, S., and ISAKA, H.: Ascitic conversion of a mixed tumor developing in rat fed with DAB. *Trans Soc Path Jap* 46: 324-325, 1957.
- (16) OPIE, E. L.: The pathogenesis of tumors of the liver produced by butter yellow. *J Exp Med* 80: 231-246, 1944.
- (17) SATO, H., and ARUJI, T.: Studies on the ascites hepatoma. *Gann* 43: 254-257, 1952.
- (18) SATOH, H.: Studies on the ascites hepatoma. XI. Difference responses by different strain of ascites hepatomas of the rats to chemotherapeutic treatment. *Gann* 47: 334-337, 1956.
- (19) YAMADA, T., and ISAKA, H.: Intercellular adhesiveness of rat ascites hepatoma cells and metastasis. *Acta Path Jap* 10: 352-353, 1960.
- (20) YOSHIDA, T.: Studies on the rat ascites sarcoma of Nagasaki strain. *Trans Soc Path Jap* 36: 3-4, 1947.
- (21) YOSHIDA, T.: The Yoshida sarcoma, an ascites tumor. *Gann* 40: 1-20, 1949.
- (22) ———: Studien über das Ascites-Hepatom. Zugleich ein Beitrag zum Begriff der cellulären Autonomie in Wachstum der malignen Geschwulst einerseits, und der Individualität der einzelnen Geschwulst anderseits. *Virchow Arch Path Anat* 330: 85-105, 1957.
- (23) ———: Screening with ascites hepatoma. *Ann NY Acad Sci* 76: 610-618, 1959.
- (24) YOSHIDA, T., SATO, H., and ARUJI, T.: Origin of the YOSHIDA sarcoma. I. Experimental production of "ascites hepatoma" in the rat. *Proc Jap Acad Tokyo* 27: 289-301, 1951.
- (25) YOSHIDA, T., ISAKA, H., NAKAMURA, K., ODASHIMA, S., and SATOH, H.: Studies on the ascites hepatoma. *Trans Soc Path Jap* 44: 407-426, 1955.
- (26) YOSHIDA, T., NAKAMURA, K., ODASHIMA, S., and ISAKA, H.: Studies on the ascites hepatoma. IX. Establishment of six further transplant strains. *Gann* 47: 612-615, 1956.
- (27) YOSHIDA, T., ODASHIMA, S., KURATA, T., NAKAMURA, K., IRAKO, Y., ISAKA, H., and ISHIZAWA, T.: Studies on the ascites hepatoma. XII. Report on establishment of nine further transplant strains. *Gann* 48: 551-553, 1957.
- (28) YOSHIDA, T., ODASHIMA, S., ISHIZAWA, T., and IRAKO, Y.: Studies on the ascites hepatoma. XV. Establishment of six new strains. *Gann* 49 (Suppl): 191-192, 1958.



## PLATES



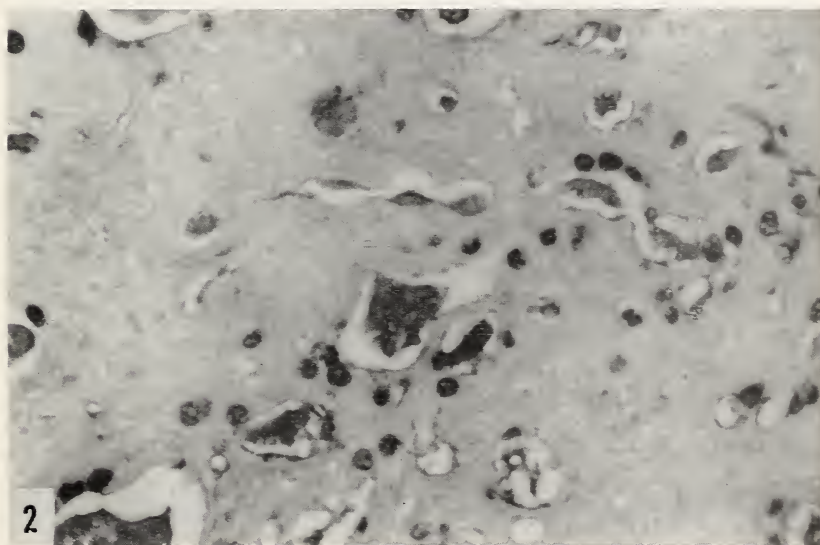
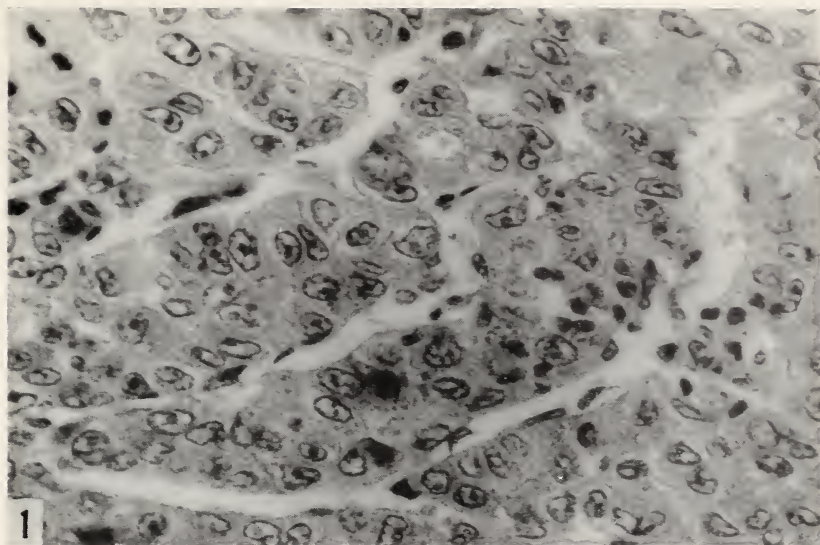


FIGURE 1.—Liver cancer (type I). Cordlike arrangement of cancer cells. Cell cords are separated from capillaries by endothelium. DAB-feeding 5 months. Killed at 277 experimental days.

FIGURE 2.—Liver cancer (type II-A). Cordlike and partially adenomatous arrangement of cancer cells including PAS-positive substance in their cytoplasm and cavities. No proliferation of connective tissues. DAB-feeding 5 months. Killed at 282 experimental days.

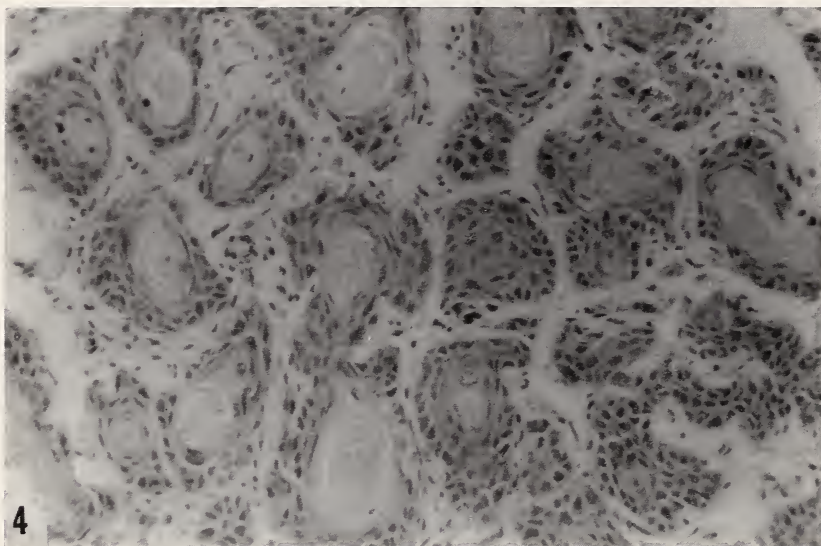
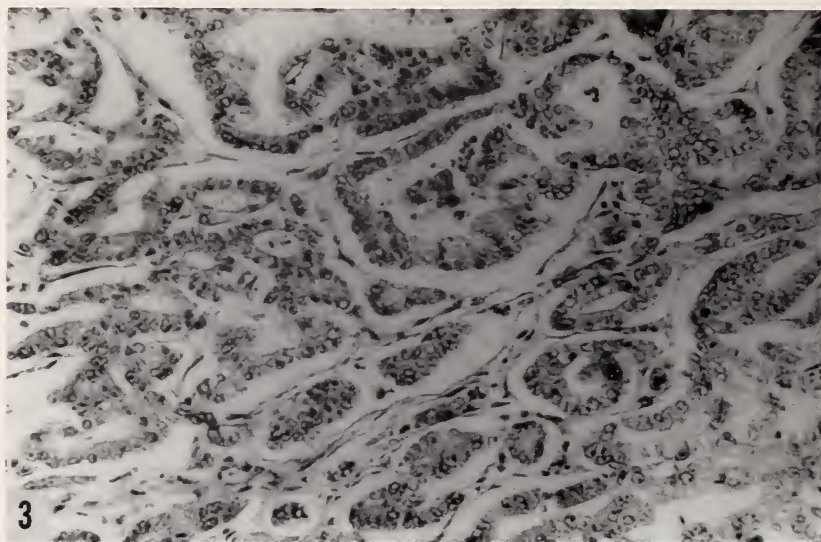


FIGURE 3.—Liver cancer (type II-B). Adenomatous structures of cancer cells separated by thin, connective, stromatic fibers. DAB-feeding 5 months. Killed at 245 experimental days.

FIGURE 4.—Squamous cell metaplasia of cancer cells found in a part of the tumor nodule from type II-B—same animal as in figure 3.

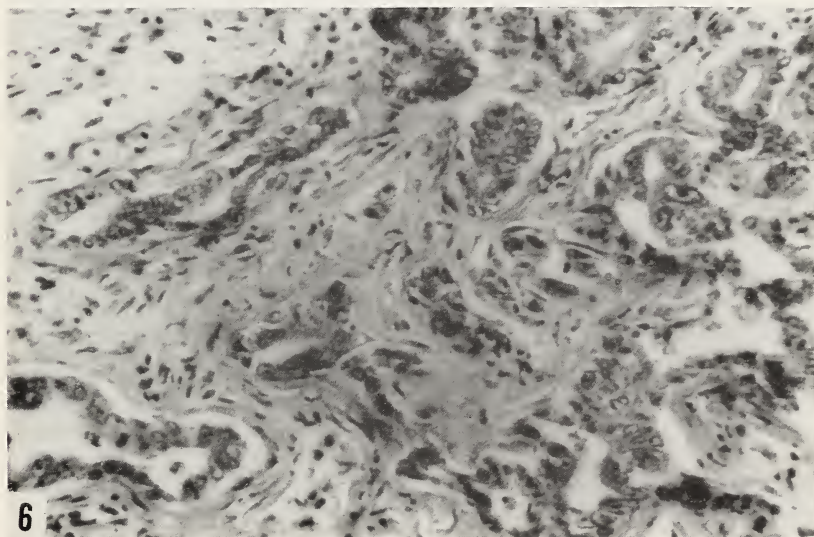
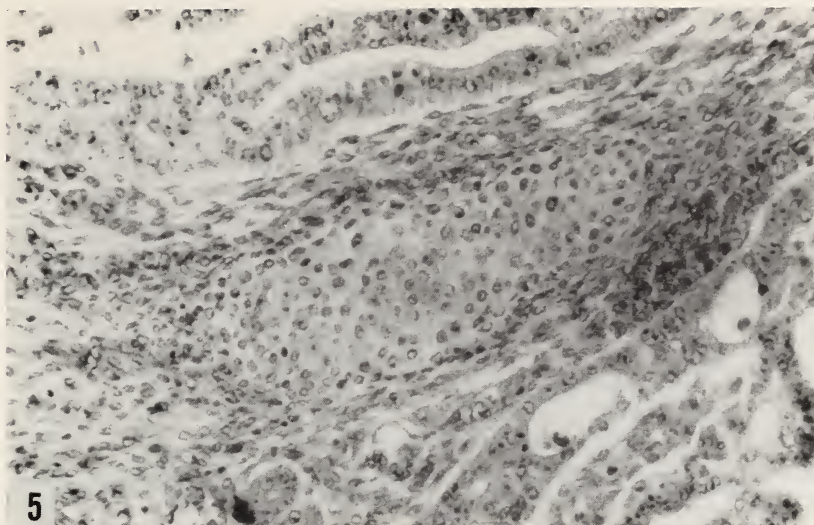


FIGURE 5.—Metaplastic cartilage formation in the fibrous connective tissues found in the tumor of type II-B. DAB-feeding 5 months. Killed at 277 experimental days.

FIGURE 6.—Liver cancer (type III). Intensive duct formation of cancer cells and marked proliferation of stromatic fibrous tissues. DAB-feeding 5 months. Killed at 270 experimental days.

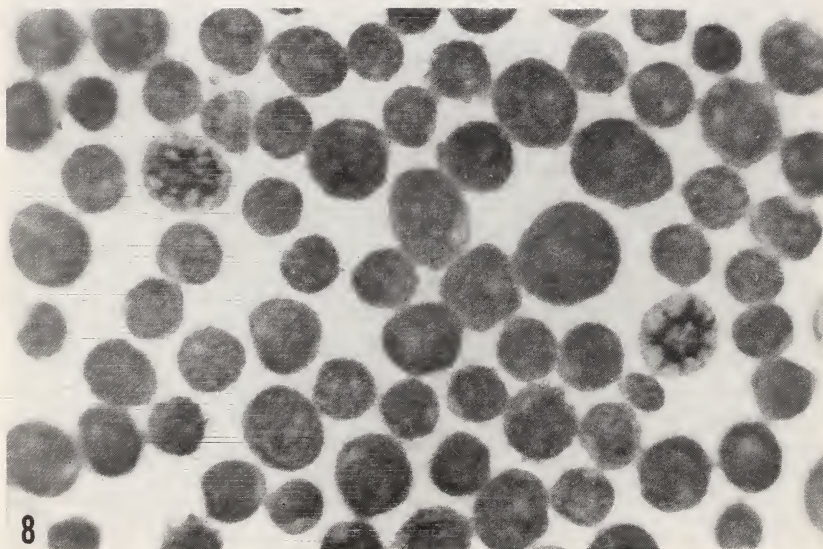
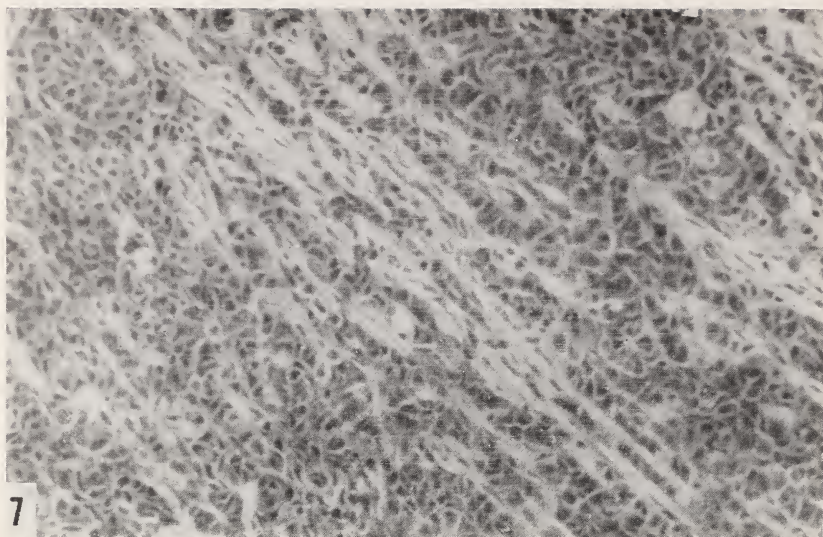


FIGURE 7.—Liver cancer (type IV). Small, irregular-shaped cancer clusters proliferating in fibrous stroma. DAB-feeding 5 months. Killed at 233 experimental days.

FIGURE 8.—Ascites picture of AH 13. A typical single cell form of ascites hepatomas. Giemsa staining.

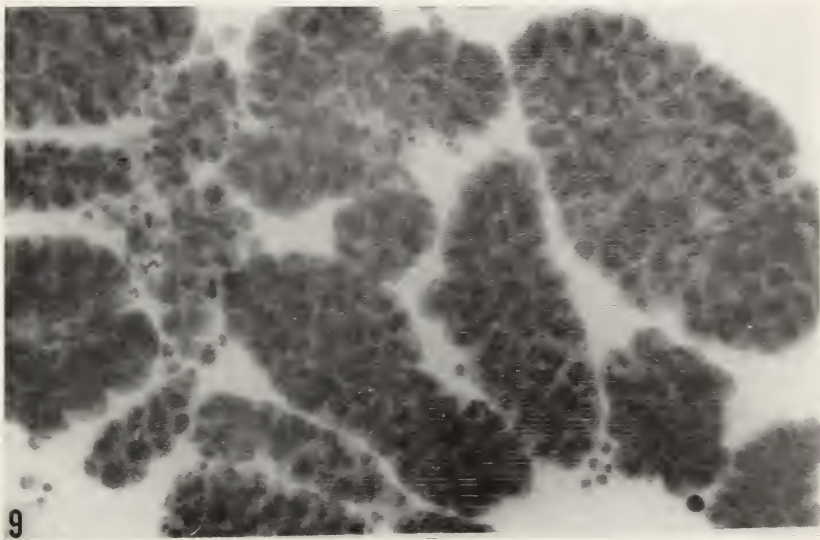


FIGURE 9.—Ascites of AH 57B. The island type of the ascites hepatoma. Stained with acetic-gentian violet solution.



## Chromosome Studies of Various Strains of Ascites Hepatomas in Rats<sup>1</sup>

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FOR many years research studies have concentrated on discovering differences between cancer cells and normal cells. Thus many workers have studied the chromosomal patterns of cancer cells, with the hope of finding abnormalities of structure and number that might elucidate the possible causal relationship of karyotypic alterations to oncogenesis. The chromosomes of cancer cells from solid tumors were first studied by section technique, but many chromosomes by this method are often split by the cutting, and the true number and configuration could not be determined.

Ascites tumors are useful for chromosomal analysis, because these tumors consist of a suspension of tumor cells. A drop of the ascitic fluid can be used to prepare a good specimen, by a simple squash technique of stained wet smears. Hence, various ascites tumors have been used frequently to study chromosomes of cancer cells. The chromosomal constitution of the Ehrlich ascites carcinoma and other ascites tumors in mice, as well as the Yoshida ascites sarcoma in rats, has been extensively studied (1, 15-18, 23, 28, 29, 36). However, almost all these ascites tumors were converted from solid tumors, either from established transplantable strains or from spontaneous ones, and they are not reproducible; therefore, a variety of tumors of common ancestry cannot be used for comparative studies of their chromosomal constitution.

The ascites hepatoma, like other ascites tumors, provides a useful tool for chromosomal analysis of cancer cells. This type of tumor is composed of "hepatoma islands" as well as individually isolated free hepatoma cells in the ascitic fluid (fig. 4). Malignant hepatomas induced in rats by feeding amino azo dyes are converted to the ascites form. The reproducibility of the ascites hepatoma distinguishes it from other ascites tumors.

Since 1951, we have established more than 50 different transplant strains of the ascites hepatoma. Each strain was derived from a different hepatoma induced in individual animals, but the normal ancestral cell of each

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was the liver cell of rats. Our preliminary work on the chromosomal patterns of 4 different strains of ascites hepatomas suggested that each tumor has its own chromosome number (11). We then examined and compared more precisely the chromosomes of 16 different strains of ascites hepatomas.

### MITOSIS OF ASCITES HEPATOMA CELLS

For comparative studies on the chromosomes of ascites hepatomas, we first had to determine the most favorable stage at which mitosis was most frequent, after transplantation of  $10^7$  tumor cells. The growth of each strain differed, as shown by varying survival times of the hosts with different tumor strains (33). Our studies revealed that for each tumor strain 4-day-old ascites was most suitable, because the ascites hepatoma was at its peak of growth, without accompanying significant degenerative changes, and many mitotic tumor cells were present.

Table 1 shows the frequency of mitotic tumor cells in 4-day-old ascites of 18 strains of ascites hepatomas. The mitotic rate was calculated by counting mitotic cells in 2,000 tumor cells examined in each strain, and the rate of each mitotic phase of the tumor cells was calculated by counting the number of cells in each mitotic phase of 200 mitotic cells. Wet smears of the suspension of tumor cells were made and stained with acetic gentian violet solution (22). The average mitotic rate ranged from 2 to 4 percent, as shown in table 1.

It was difficult to detect a significant difference in the mitotic rates of the 18 strains. The frequency of cells in metaphase in the mitotic cells was 18 to 42 percent, greater than that of anaphase or telophase. This may suggest that the rate of each mitotic phase is in proportion to the rate of duration of the corresponding mitotic phase in the entire mitotic time. But phase contrast microscopic observations of the *in vitro* mitotic process of living ascites hepatoma cells showed that the longer the time of each mitotic phase, the larger its number in the ascites specimen.

For phase contrast microscopy of mitotic cells, a drop of ascitic fluid taken 3 to 24 hours after transplantation of the tumor was placed on a clean glass slide, covered with a clean coverslip, and then sealed with paraffin or balsam-paraffin. The preparation was examined under the phase contrast microscope in a thermostable chamber regulated at 37° C, at a magnification of 900 to 1,000. The mitotic time and the time of each mitotic phase of strains AH 7974 and AH 130 measured by this method are shown in tables 2 and 3 (9, 13). In these tables the time of prophase is indicated by the measured time plus X. X is the unmeasured time from the beginning of the prophase to the moment the observation was started. The mitotic time of AH 7974 cells and AH 130 cells was less than 1 hour, but the mitotic time of the former was longer than that of the latter. Metaphase was shorter than that of telophase, but longer than that of anaphase; in some specimens metaphase was shorter than anaphase.

TABLE 1.—Frequency of mitosis in 4-day-old ascites of 18 strains of ascites hepatomas

Strains	Mitotic rate among 2,000 cells (%)		Rate of each mitotic phase among 200 mitoses (%)									
			Prophase		Prometaphase		Metaphase		Anaphase		Telophase	
	Mean	(min-max)	Mean	(min-max)	Mean	(min-max)	Mean	(min-max)	Mean	(min-max)	Mean	(min-max)
AH 149	2.0	(1.6-2.4)	35.0	(25.5-44.0)	11.6	(8.5-16.5)	34.4	(30.0-39.5)	4.1	(1.0-8.0)	14.9	(9.5-17.0)
AH 39	2.2	(1.6-2.7)	28.1	(23.0-34.0)	21.0	(16.5-28.0)	22.7	(20.0-27.0)	5.1	(3.5-7.5)	23.1	(22.5-24.5)
AH 13	2.3	(1.9-2.6)	12.6	(9.0-16.0)	15.8	(12.0-21.0)	41.8	(37.0-46.0)	7.4	(4.0-12.0)	22.4	(19.0-24.0)
AH 130	2.3	(2.0-2.6)	34.9	(31.0-39.0)	11.3	(6.0-19.0)	27.6	(24.0-31.5)	4.5	(4.0-6.5)	21.7	(12.0-32.5)
AH 7974	2.4	(2.4-2.6)	17.8	(14.0-20.0)	13.8	(8.0-19.0)	35.2	(32.0-37.0)	7.8	(7.0-9.0)	25.4	(21.0-29.0)
AH 21	2.4	(2.0-3.1)	17.4	(10.0-28.0)	20.6	(18.0-26.0)	34.4	(26.0-40.0)	9.6	(6.0-13.0)	18.0	(15.0-22.0)
AH 66F	2.4	(1.8-3.4)	36.8	(29.5-43.5)	13.3	(10.0-16.5)	32.8	(29.5-34.5)	3.6	(2.0-5.5)	13.5	(8.5-20.0)
AH 318	2.6	(2.0-3.1)	32.3	(29.5-37.5)	19.5	(15.0-24.5)	18.2	(16.5-22.0)	5.4	(4.5-6.5)	24.6	(19.5-32.5)
AH 49	2.8	(2.4-3.4)	14.5	(10.5-18.0)	15.6	(10.0-22.0)	39.4	(32.0-44.0)	7.2	(4.0-9.0)	25.0	(21.0-26.0)
AH 63	3.0	(2.5-3.4)	26.0	(23.0-29.5)	24.1	(17.5-30.0)	25.0	(19.5-32.5)	4.6	(3.0-6.5)	20.3	(17.5-23.5)
AH 423	3.0	(2.4-3.3)	15.3	(11.0-20.5)	9.8	(7.0-13.5)	34.6	(31.0-43.5)	19.3	(14.5-26.0)	21.0	(12.0-30.0)
AH 601	3.1	(2.3-3.7)	19.3	(13.5-24.0)	16.7	(11.0-26.0)	29.2	(20.5-33.0)	15.5	(8.0-22.5)	15.5	(13.5-22.0)
AH 364	3.2	(1.4-4.4)	24.0	(16.0-27.5)	8.8	(3.5-11.5)	28.5	(23.5-37.0)	14.2	(11.5-16.0)	24.5	(20.0-27.5)
AH 66	3.4	(3.1-4.0)	24.3	(21.0-30.5)	14.4	(10.5-19.8)	16.7	(13.0-23.0)	18.7	(13.5-19.5)	25.3	(18.5-32.5)
AH 414	3.5	(2.0-5.2)	23.6	(21.0-27.0)	12.8	(11.5-15.0)	25.9	(24.0-28.5)	15.4	(13.5-18.5)	22.4	(19.5-26.5)
AH 99	3.8	(3.5-4.4)	26.7	(22.0-32.5)	9.8	(6.0-10.5)	28.9	(26.0-33.0)	14.0	(12.0-15.0)	20.7	(18.5-24.5)
AH 322	3.9	(2.6-4.6)	22.6	(15.0-24.5)	9.7	(4.5-14.0)	29.1	(23.0-34.5)	14.8	(8.5-23.0)	23.0	(19.5-29.5)
AH 602	4.0	(2.9-4.5)	19.6	(16.0-22.5)	18.3	(10.0-33.5)	22.5	(17.0-26.5)	18.8	(13.5-23.5)	22.8	(18.5-27.5)

TABLE 2.—Course of time of mitosis of AH 7974 cells, by phase contrast microscopy\*

Case	Phase (min)				Total
	Prophase	Metaphase	Anaphase	Telophase	
1	X <sub>1</sub> + 16	14	3½	24	X <sub>1</sub> + 57½
2	X <sub>2</sub> + 13	9	3	16	X <sub>2</sub> + 41
3	X <sub>3</sub> + 18	8	4	12	X <sub>3</sub> + 42
4	X <sub>4</sub> + 15	3	3	29	X <sub>4</sub> + 50
5	X <sub>5</sub> + 5	3	2	17	X <sub>5</sub> + 27
6	X <sub>6</sub> + 9½	8	2	15	X <sub>6</sub> + 34½
Average					X + 42

\*X = unmeasured time from the beginning of prophase to the moment when the observation was started.

TABLE 3.—Course of time of mitosis of AH 130 cells, by phase contrast microscopy\*

Case	Phase				Total
	Prophase	Metaphase	Anaphase	Telophase	
1	X <sub>1</sub> + 9 min 50 sec	2½ min	5 min 55 sec	14 min 35 sec	X <sub>1</sub> + 32 min 50 sec
2	X <sub>2</sub> + 3 min 25 sec	5½ min	1 min 40 sec	15 min 40 sec	X <sub>2</sub> + 26 min 15 sec
3	X <sub>3</sub> + 10 min	2½ min	6 min	10 min	X <sub>3</sub> + 28 min 30 sec
4	X <sub>4</sub> + 2 min 45 sec	5½ min	1 min 40 sec	14 min 35 sec	X <sub>4</sub> + 24 min 30 sec
Average					X + 28 min 1 sec

\*X = unmeasured time from the beginning of prophase to the moment when the observation was started.

From the 2d to the 4th day after transplantation of AH 130 cells, their active growth in ascites was indicated by a constantly increasing linear curve. At this stage, the ascites of AH 130 could be used for the mitotic time and the generation time of its cells, which were estimated mathematically by the following formulas of Sato *et al.* (26):

$$Q = P \left( 1 + \frac{A + B}{2} \right)^{\frac{24}{m}}, \quad Q = P \times 2^{\frac{24}{m + p}}$$

- P: total number of tumor cells per mm<sup>3</sup> ascites 3 days after transplantation;  
Q: total number of tumor cells per mm<sup>3</sup> ascites 4 days after transplantation;  
A: percentage of mitotic cells in 3d-day ascites;

*B*: percentage of mitotic cells in 4th-day ascites; *m*: mitotic time; *P*: intermitotic time. The mitotic time (*m*) and the generation time ( $m + p$ ), thus estimated, averaged 50 minutes and 20 hours, respectively.

The phase contrast microscopy of mitotic ascites hepatoma cells showed the following abnormal mitotic divisions of cells: 1) A chromosome bridge was observed in anaphase with no following telophase, but, after the bridged chromosome moved to one of the separated chromosome groups, each established a new nuclear membrane returning to a resting nucleus stage, with a resultant binucleated tumor cell. 2) In the prophase and metaphase, chromosomal separation was suppressed and the nucleus returned to the resting stage. 3) In the telophase the furrow which appeared at the periphery was obliterated, but each group of chromosomes reconstructed a resting nucleus. Finally, a binucleated tumor cell resulted. 4) In the multipolar divisions the tripolar mitosis type was relatively frequent. 5) Immediately after the formation of 2 daughter cells, they fused again and a binucleated cell was formed. Similar findings with Yoshida sarcoma cells have also been reported by Hirono (5) and others (24, 35).

Mitotic tumor cells belonging to the hepatoma island frequently were located on the outer surface of the island. One of the resulting daughter cells separated freely from the mother island, and the other one remained at the island. In another instance, it was noted that both daughter cells remained at the mother island (9). It has not been observed that both daughter cells separated from the island. Mitotic-free cells caused free daughter cells or "doubles," the clusters of 2 cells. However, carefully repeated observations have never demonstrated that free daughter cells adhere anomalously to different neighboring tumor cells or islands, *i.e.*, free daughter cells only adhere to their mother cell islands, at their sister cells, or their free sister cells.

## EXAMINATION OF CHROMOSOMES

The ascites hepatoma cells of the rat have a large number of chromosomes that are usually crowded on the nuclear plate. Therefore, the determination of the number and structure of individual chromosomes is not always easy. A previous study (6) indicated that a hypotonic solution facilitates the spreading of chromosomes, and that excellent specimens could be obtained by adding a large volume of hypotonic (0.5%) NaCl solution to the ascites hepatoma cells before staining. We pre-treated the cells of strains AH 130, AH 7974, AH 601, AH 602, and AH 66 by a similar method: Thirty ml of a 0.5 percent NaCl solution was injected intraperitoneally immediately after the 4-day-old tumor animals had been sacrificed. The peritoneal cavity was then opened and the diluted ascitic fluid placed in a tube and centrifuged. A large amount of acetic gentian violet, or orcein, solution was added to the sedimented

tumor cells and examined. This procedure was completed within 30 minutes. The cells of strain AH 414 were surprisingly resistant to treatment with hypotonic NaCl solution and spreading of their chromosomes was unsuccessful. Therefore, we re-examined other methods for spreading the chromosomes.

Tests were performed *in vitro* with various hypotonic solutions—special consideration being given to the type of solution, its concentration, and the time of treatment. A pure culture of ascites tumor was divided into 15 equal volumes and mixed separately with 5 different concentrations of hypotonic NaCl, MgCl<sub>2</sub>, and CaCl<sub>2</sub> solutions. The ratio of ascites to the hypotonic solution was 1:20 by volume. The mixed suspension of tumor cells was agitated at 37° C for 5 to 15 minutes, stained with acetic orcein, and examined microscopically to determine whether chromosomes in metaphase were adequately spread. The chromosomes of AH 414 cells were well spread by a hypotonic MgCl<sub>2</sub> solution (table 4).

TABLE 4.—Hypotonic solutions favorable for spreading chromosomes of ascites hepatoma cells *in vitro*

Strains	Hypotonic solutions	Concentration (M)	Treatment (min)
AH 414	MgCl <sub>2</sub>	0.04	10
AH 272	MgCl <sub>2</sub>	0.04	10
AH 310	NaCl	0.03–0.04	
AH 423			
AH 130			
AH 7974			
AH 601			
AH 602			
AH 66			
AH 39	NaCl	0.03–0.04	10
AH 99			
AH 66F			
AH 322			
AH 13			
AH 62			
AH 62F			

Based on these results, other specimens for chromosomal analysis were prepared as follows: Ascitic fluid from 4-day-old rats with ascites hepatomas was mixed in a test tube with a 0.03 or 0.04 M NaCl or MgCl<sub>2</sub> solution at 37° C, in a ratio of 1:20 of the ascites-hypotonic solution. After the contents of the test tube were well shaken, the tube was kept at 37° C for 5 to 15 minutes, then immediately centrifuged, and the supernatant fluid decanted. The sedimented tumor material was stained with acetic orcein—or gentian violet—solution, about 3 to 5 times the volume of the tumor material, and stirred with a glass rod. A drop of this mixture was placed on a glass slide, covered with a coverslip under adequate pressure, and then sealed with balsam-paraffin. This method was used to prepare specimens of tumors of AH 414, AH 272, AH 310, AH 423, AH 39, AH 99, AH 322, AH 13, AH 62, AH 62F, and AH 66F.

All metaphase plates of mitotic cells observed microscopically at  $2,000\times$  were examined because it was considered essential to study all chromosomal pictures. One hundred plates were examined for each tumor strain. All plates were drawn with Hamano's projection apparatus attached to one of the eye lenses of the binocular microscope used, and photographs of all the observed plates were taken simultaneously with a 35 mm camera connected to the tube of the same microscope (figs. 1 and 2). The number of chromosomes and their morphological characteristics were carefully studied in each tumor strain.

## CHROMOSOMES OF ASCITES HEPATOMA CELLS OF DIFFERENT STRAINS

### Ascites Hepatomas Examined

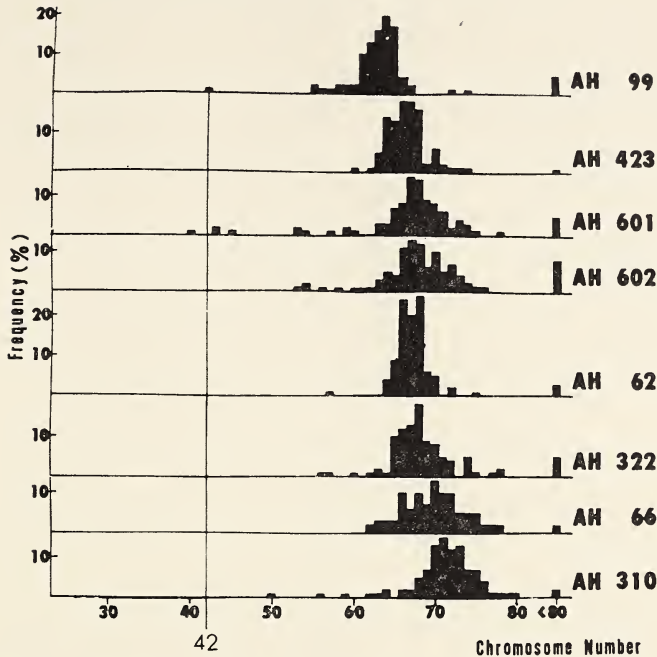
We have studied the chromosomes of 16 different strains and some of the results have been briefly reported (20, 34). The tumor strains examined are listed in table 5. These strains vary considerably in their biological characteristics, such as growth rate, transplantability, ascites, and sensitivity to some chemotherapeutic agents and to X irradiation (30-33). The two sublines AH 601 and AH 602 were converted into the ascites form from hepatomas after various transplant generations (7). AH 66F is a subline of AH 66, which was derived from one of the stock transfer lines of the mother AH 66 after the ascites changed abruptly (33). AH 62F is also a subline of AH 62, developed after the AH 62 cells were kept at  $-80^{\circ}\text{C}$  for 111 days. The characteristics of this subline are also quite different from those of the parent line AH 62 (33). The remaining 10 strains are descendants of different induced tumors. The original tumors of the present 16 ascites hepatomas were developed in Japanese common albino rats and serially transplanted in "Donryu" rats—a highly susceptible, closed colony of our own breed—in this laboratory.

### Chromosome Numbers

Table 6 and text-figures 1 and 2 show the chromosome number of the 16 strains of ascites hepatomas. No individual number of chromosomes absolutely predominated in each tumor strain. Usually the number was distributed in a wide range, showing one modal variation. The modal chromosome number was: 38 in AH 66F and AH 13; 43 in AH 130; 44 in AH 39; 48 in AH 62F; 49 in AH 7974; 59 in AH 272; 64 in AH 99; and 68 in AH 322. In the remaining 7 strains, however, no predominant chromosome number was demonstrated in an examination of 100 metaphases, but the most frequent numbers ranged from 45-48 in AH 414; 64-68 in AH 423; 67-68 in AH 601; 66-68 in AH 602; 66-68 in AH 62; 66-72 in AH 66; and 70-74 in AH 310. This suggests that each tumor strain has its own modal chromosome number, which varies in each strain.

TABLE 5.—Characteristics of 16 strains of ascites hepatomas

Strains	Takes (%)	Survival (days)	Ascites (type)	Invasion	Metastasis	Nitromin sensitivity MED <i>in vivo</i> (mg/kg)
AH 66F	89	10	Free cell	Slight	Frequent	1.0
AH 13	96	7	Free cell	Slight	Frequent	1.0
AH 130	97	12	Free cell + island	Intensive	Frequent	1.0
AH 39	90	14	Free cell	Moderate	Seldom	7.5
AH 414	80	16	Free cell	Moderate	Occasional	10.0
AH 62F	82	18	Free cell	Moderate	Seldom	20.0
AH 7974	97	12	Island, small	Intensive	Frequent	50.0
AH 272	99	6	Free cell + island	Slight	Occasional	1.0
AH 99	92	10	Island, middle	Moderate	Occasional	1.0
AH 423	75	14	Free cell	Slight	Seldom	50.0
AH 601	87	15	Island, middle	Slight	Occasional	1.0
AH 602	94	19	Island, middle	Moderate	Frequent	10.0
AH 62	95	11	Island, middle	Moderate	Frequent	20.0
AH 322	87	11	Island, middle	Moderate	Frequent	50.0
AH 66	93	11	Free cell + island	Slight	Occasional	50.0
AH 310	85	13	Island, small	Slight	Seldom	25.0



TEXT-FIGURE 1.—Histogram showing the frequency of cells with various chromosome numbers in 8 strains of ascites hepatomas.

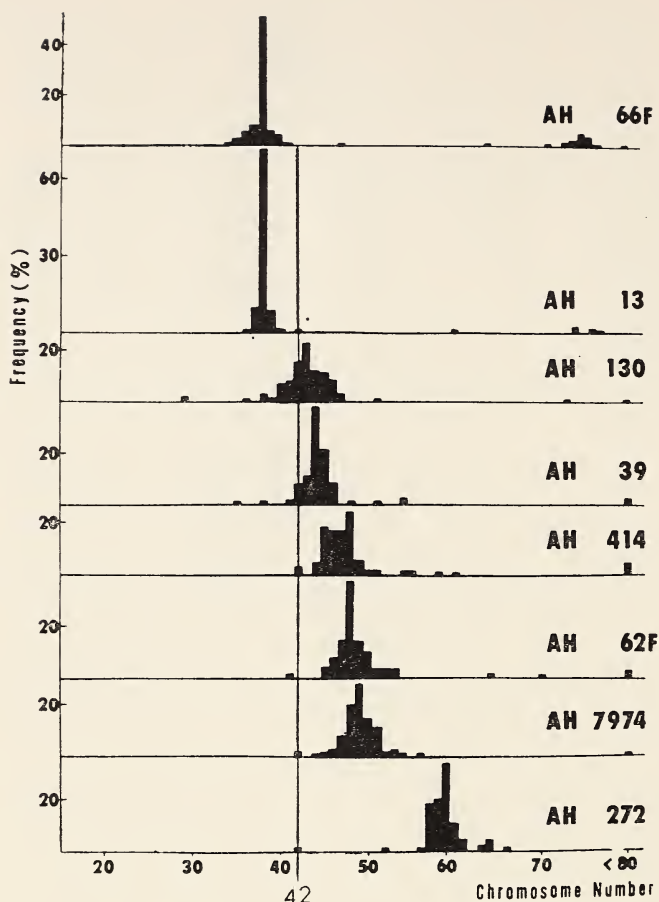
There was no difference in the modal chromosome number of AH 66F and AH 13, but the range of chromosome number appeared different: AH 13 fluctuated around 38 within a very narrow range, while that of AH 66F fluctuated within a rather wide range. It may be safe to say that the range of variation in the chromosome number is to some extent dependent on the ploidy or the modal chromosome number, *i.e.*, with the larger chromosome number of the mode there tends to be a greater range of variation in chromosome number. An exception was the chromosome number distribution of AH 601 and AH 602 which showed a similarity. There were no ascites hepatomas with the modal chromosome number of 42, the chromosome number of germ cells or liver cells in the rat (27, 29). It may be also noteworthy that the chromosome number falls between the hypodiploid and hypotetraploid. There was no parallelism found between the size of tumor cells and their modal chromosome numbers when the diameter and chromosome number of tumor cells of different strains were studied (table 7).

### Chromosome Morphology

Figure 5 shows idiograms of typical chromosomes in the modes or chromosomes in the midregions of the high prevailing numbers of the 14 strains of ascites hepatomas examined. Chromosomes of different shapes were seen, but the main types were classified morphologically into meta-







TEXT-FIGURE 2.—Histogram showing the frequency of cells with various chromosome numbers in 8 strains of ascites hepatomas.

centric (M), submetacentric or subtelocentric (S), and telocentric (T) according to the location of the centromere, similar to the normal liver cell of the rat (29). Occasionally, it was not easy to distinguish between M and S chromosomes, especially in small ones. In such instances the length of both arms of the chromosomes was measured by 3 persons and, when the length ratio of the arms of the chromosomes was in the range from 1:1 to 1:1.3, they were classified into M groups. It should be emphasized that all attempts to bring chromosomes of each strain of the ascites hepatoma into analogous pairs failed.

Large M chromosomes more than 1.5 times longer than the following M chromosomes were detected in only 5 strains, but their number and size varied by strain as shown in figure 5. There was 1 large M chromosome in AH 66F, AH 13, AH 272, and AH 423, while there were 3 of different lengths in AH 99. The chromosomes of each tumor strain were arranged in a declining order of chromosome length, and the large

TABLE 7.—Size of living ascites tumor cells

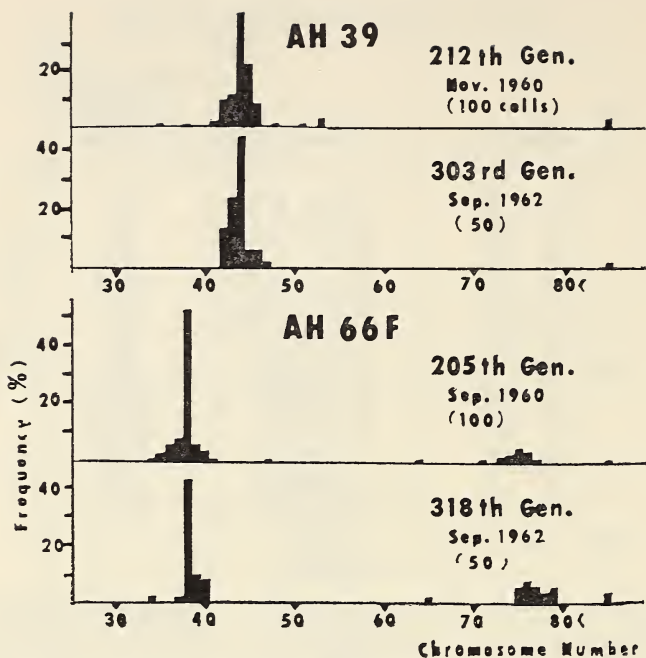
Strains	Diameter of cells ( $\mu$ )	Modal or high prevailing chromosome numbers
AH 66F	15.1 $\pm$ 2.5	38
AH 13	12.9 $\pm$ 1.7	38
AH 130	12.6 $\pm$ 1.9	43
AH 39	12.9 $\pm$ 2.3	44
AH 414	13.1 $\pm$ 2.7	45-48
AH 62F	12.7 $\pm$ 1.8	48
AH 7974	12.7 $\pm$ 2.2	49
AH 272	13.0 $\pm$ 1.7	59
AH 99	11.5 $\pm$ 1.6	64
AH 423	13.6 $\pm$ 2.3	64-68
AH 601	11.5 $\pm$ 1.9	67-68
AH 602	10.0 $\pm$ 1.6	66-68
AH 62	11.8 $\pm$ 2.7	66-68
AH 322	12.2 $\pm$ 2.7	68
AH 66	13.8 $\pm$ 2.5	66-72
AH 310	12.0 $\pm$ 2.4	70-74

M chromosomes occupied various positions. Small rod-shaped chromosomes were found in only 5 strains: AH 66, AH 66F, AH 310, AH 322, and AH 7974. This chromosome is indicated by m in the idiograms (fig. 5) and in the microphotograph of the AH 66F cell (fig. 2). The SAT chromosome (fig. 5) seen only in AH 310 differed markedly in shape from S chromosomes. The SAT chromosome is composed of a rod and minute body, both parts being connected by a fairly long, fine thread (fig. 5). An outstandingly large S chromosome was detected in AH 66F, AH 13, and AH 99, and it was the longest one in each idiogram of these 3 strains. Each of the 4 types of chromosomes, *i.e.*, the large M and S, m, and SAT, never found in the normal liver cell of rats and whose origin is unknown, was detected in more than 60 percent of all the metaphases of these strains. Any of these characteristic chromosomes, however, were uncommon in all the ascites hepatomas examined. There were some numerical variations even in the nuclear plates of modal regions of the same tumor. These observations may suggest that each strain of the ascites hepatoma has its own chromosomal pattern.

The endomitotic reduplication, which was well described by Levan and Hauschka (16), was seen rarely in cells of AH 39 and AH 310. Figure 6 shows a metaphase of the endomitotic reduplication.

#### Stability and Variability of Chromosome Constitutions

The chromosomal pattern of strains AH 39 and AH 66F was examined twice at different transfer generations at 2-year intervals. The results are shown in text-figure 3. The number and morphology of chromosomes of different transfer generations of each tumor showed no differences. This chromosome constitution of tumors remained stable during their



TEXT-FIGURE 3.—Histogram showing the stability in the chromosome constitution of ascites hepatomas AH 39 and AH 66F, analyzed at 2-year intervals.

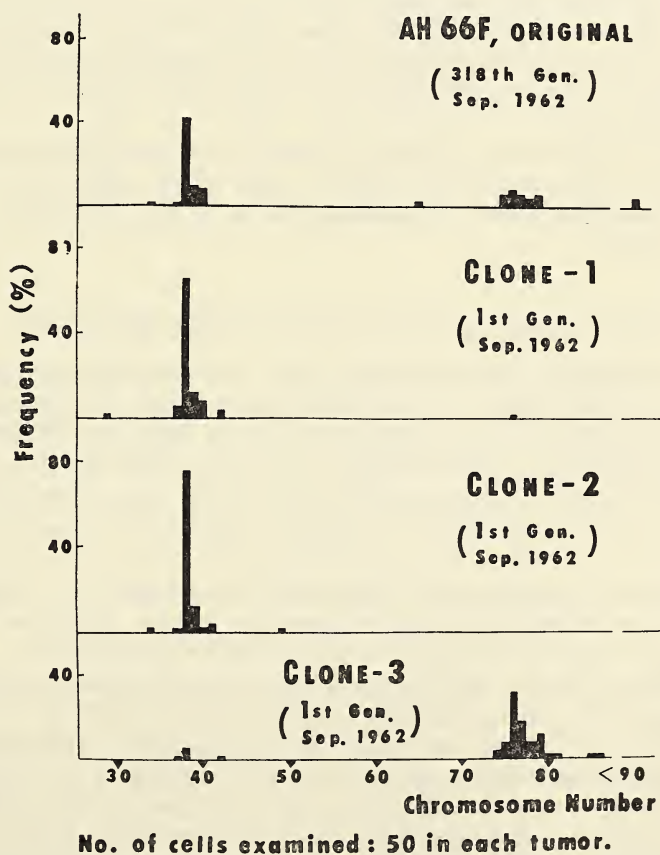
serial animal passages. As indicated, strain AH 66F is a subline tumor derived from one of the stock transfer lines of AH 66, and the chromosome number and general characteristics of both tumors differ (text-fig. 1). Therefore, the chromosome constitution of tumor AH 66 has changed during growth, though chromosomal constancy has been demonstrated in its subline AH 66F. Similar chromosomal changes occurred in AH 62 resulting in the appearance of substrain AH 62F (text-fig. 2 and fig. 5).

#### Population Analysis

Interestingly, AH 66F, a hypodiploid tumor, contained many polyploid cells comprising about 10 percent of the whole tumor cell population when first examined, but which increased to 30 percent after 2 years of serial transfer (text-fig. 3). Most of the polyploid cells have 76, or nearly 76, hypotetraploid chromosomes, double the number of the predominant modal chromosome number of this strain. Such a complexity in chromosome numbers within the tumor cell population of AH 66F led to an attempt to establish clonal tumors from the AH 66F strain by single cell transplantation to compare the chromosomes of both clones and the parent line AH 66F. The single cell transplantation procedure was as follows: The ascites of AH 66F (fig. 3) containing well-proliferated tumor cells was mixed with varying dilutions (always more than 50,000 times) of a mixture of horse serum and Hanks' balanced saline solution so that a drop

of the diluted ascitic fluid contained only one or a few cells. A drop of the diluted suspension of tumor cells was put on a clean coverglass which was gently placed over a moist hanging-drop chamber and the cells were counted microscopically at a magnification of 80 to 320. With a micro-manipulator a drop having only one cell was drawn into a microcapillary glass tube, and enough of the diluting fluid was added to make the volume sufficient for the usual transplantation technique. This was then injected intraperitoneally into a normal Donryu rat by puncturing the abdominal wall with the microcapillary. The ascitic fluid was examined at 4-day intervals for tumor cells.

Comparative studies on the chromosomes of AH 66F and its single cell clones demonstrated that the chromosome number and the chromosomal pattern of 2 clonal populations developed, *i.e.*, clone-1 and clone-2 showed complete conformity to those of the predominant modal region of the parent line, while the remaining one, clone-3, revealed the same chromosome number and karyotype as those of hypotetraploid cells of the parent line (text-fig. 4) (8). This may imply that each of these clones may have



TEXT-FIGURE 4.—Histogram showing the chromosome number distribution of ascites hepatoma AH 66F and its 3 single cell clones.

been derived from a ploidy-different single hypodiploid or hypotetraploid cell of the parent line population. Hence, one may have a "mosaic concept" of neoplastic population (2-4). Studies on the stability of these chromosomally different clones are now being investigated.

In other experiments with the Yoshida sarcoma (14), a near-diploid rat tumor with 40 elements in the modal chromosome, a polyploid clonal subline has also been established successfully from the stock line, and the chromosome number of the clone—80 in the modal number—has persisted during 2 years of animal passage over 200 transfer generations. No detectable, significant differences between the clone and its parent line were found, except for the chromosome constitution and the size of tumor cells in such aspects as transplantability, growth rate, ability to metastasize, and sensitivity to alkylating agents. The tumor cells ( $10^5$ ) of each line were mixed and transplanted into the abdominal cavity of a rat. The tumor ascites which developed showed that the cell population in the ascites was a mosaic mixture of near-diploid and near-tetraploid cells with a ratio of 1:1. However, the former cells predominated in 6 further transfer generations, displacing the latter about 5 percent. These observations may support the fact that some of the tumor cells with a double number of chromosome sets can grow successfully, but they cannot predominate in the original tumor cell population because of their low proliferative power.

Each of the hypodiploid clones of AH 66F established showed the modal chromosome number of 38, the modal number of the parent line. Similar findings were obtained by our previous studies on the population analysis of AH 130 (12).

### Chromosome Number Variation in Clonal Populations

The chromosome number of clonal tumor cell populations varied even in the very early stage of their development. An unusual study was reported by Nakamura (19), with the hepatoma islands of AH 130. The ascites of this strain contains abundant free tumor cells and a few islands (fig. 4). By *in vitro* treatment with trypsin, about 99 percent of the tumor cells were freely isolated—the remaining 1 percent consisted of small islands of 2 or 3 cells. Increasing number of islands of various sizes appeared after transplantation of trypsinized tumor cells (25). Nakamura examined the difference in the chromosome number of tumor cells of individual islands that developed 48 hours after transplantation. Almost all the islands present were examples of naturally prepared clones, each the aggregate of cells derived from a separated individual cell. This was demonstrated by the results of single cell transplantation of the tumor (10) and phase contrast microscopy of living AH 130 cells, as described previously. Even between two cells of a "double," a union of 2 cells, there was a difference in their chromosome numbers, the largest one noted being 6, and the difference did not increase regularly with the growth of the islands through further repetition of mitosis of island cells.

We recently performed a similar study and the results, which were principally the same as Nakamura's, are shown in table 8. Figures 7, 8, and 9 show the chromosome idiograms of tumor cells of the same islands with differences in their chromosome numbers. Figure 10 presents the chromosomal patterns of the same island cells with no difference.

TABLE 8.—Chromosome numbers of tumor cells belonging to hepatoma islands of various sizes—strain AH 130

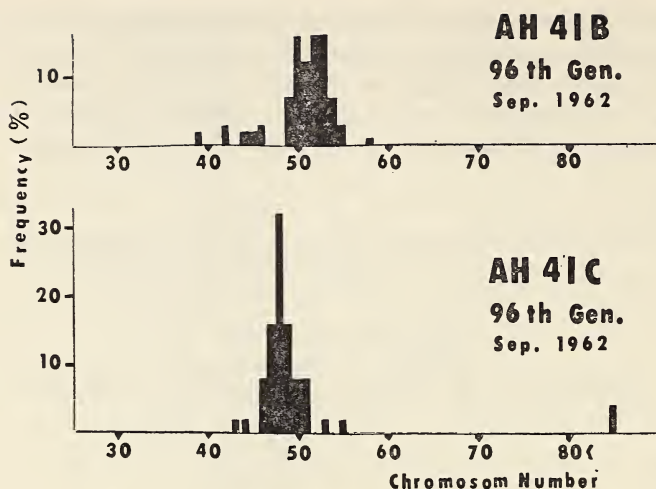
Island No.	Number of cells constituting the islands	Chromosome No. examined		Difference	
1	11	43	43	0	
2	16	43	43	0	
3	23	43	43	0	
4	3	45	45	0	
5	6	45	45	0	
6	11	46	46	0	
7	12	43	44	1	
8	19	42	43	1	
9	8	40	42	2	
10	11	45	45 46	0	1 1
11	5	48	49	1	
12	3	43	45	2	
13	8	43	45	2	
14	4	45	47	2	
15	8	43	47	4	
16	9	44	51	7	

#### Tumor Cells With Smaller Numbers of Chromosomes

During this investigation, we observed tumor cells in several strains of ascites hepatomas with a small number of chromosomes (figs. 11, 12, 13, and 14). The smallest chromosome number observed was 2, but the frequency was low and we believe that they may be products of some abnormal mitotic cells. It would be of interest to determine their viability as they have entered mitosis and reached metaphase.

#### Chromosomes of Different Ascites Hepatoma Strains From Separate Primary Hepatoma Nodules From the Same Rat

Recently, Odashima, in our laboratory, has successfully established 2 strains of ascites hepatomas. They were derived from different primary hepatoma nodules that developed in separate liver lobes of the same Donryu rat fed 0.06 percent *N,N*-dimethyl-*p*-(*m*-tolylazo)aniline for 180 days (21). They were designated as strains AH 41B and AH 41C. They varied in their chromosome number as well as other characteristics such as ascites, ability to metastasize, and drug sensitivity (text-fig. 5 and table 9).



No. of cells examined ; 50 each.

TEXT-FIGURE 5.—Histogram showing the chromosome number of ascites hepatomas AH 41B and AH 41C, descendants of separate hepatomas from the same rat.

TABLE 9.—Two different strains of ascites hepatomas derived from separate primary hepatomas in the same rat

	AH 41B	AH 41C
Takes (%)	90	95
Survival time of hosts (days)	12	10
Diameter of cells ( $\mu$ )	$12.9 \pm 2.8$	$12.1 \pm 1.6$
Ascites (type)	Island	Free cell
Metastasis	Moderate	Frequent
Nitromin sensitivity (MED <i>in vivo</i> , mg/kg)	25	5

## REFERENCES

- (1) BAYREUTHER, K.: Der Chromosomenbestand des Ehrlich-Ascites-tumors der Maus. *Z Naturforsch* 7: 554-557, 1952.
- (2) HAUSCHKA, T. S.: Methods of conditioning the graft in tumor transplantation. *J Nat Cancer Inst* 14: 723-739, 1953.
- (3) ———: Tissue genetics of neoplastic cell populations. *Canad Cancer Conf* 2: 305-345, 1957.
- (4) ———: Correlation of chromosomal and physiologic change in tumors. *J Cell Comp Physiol* 52 (Suppl): 197-233, 1958.
- (5) HIRONO, I.: Some observations on the mitosis of the living malignant tumor cells. Studies on Yoshida sarcoma cells. *Acta Path Jap* 1: 40-47, 1951.

- (6) HSU, T. C., and POMERAT, C. M.: Mammalian chromosomes in vitro. III. A method for spreading the chromosomes of cells in tissue culture. *J Hered* 44: 23-29, 1953.
- (7) ISAKA, H.: Transformation of the OAT-hepatoma into ascites form. *Gann* 44: 174-176, 1953.
- (8) ———: On the chromosome of ascites tumors. Presented in the symposium "Characteristics of Cancer Growth" of special autumn meeting of Jap Path Soc 1962, November.
- (9) ISAKA, H., NAKAMURA, K., and KAZIWARA, K.: Studies on the ascites hepatoma. III. Phasemicroscopical observation of mitotic process of tumor cell. *Trans Soc Path Jap* 42: 407-408, 1953.
- (10) ISAKA, H., NAKAMURA, K., and ODASHIMA, S.: Studies on the ascites hepatoma. V. Transplantation of ascites hepatoma with a single cell. *Gann* 45: 434-436, 1954.
- (11) ———: Studies on the ascites hepatoma. VI. On the chromosomes of ascites hepatoma cells. *Gann* 45: 172-174, 1954.
- (12) ———: Studies on the ascites hepatoma. VII. On the chromosome number of the ascites hepatoma cells derived from a single cell. *Gann* 46: 194-196, 1955.
- (13) ISAKA, H., NAKAMURA, K., ODASHIMA, S., and SATOH, H.: Unpublished data.
- (14) ISAKA, H., SATOH, H., and OISHI, Y.: Studies on the chromosomes of two different sublines of Yoshida ascites sarcoma; a polyploid subline and an  $\text{HN}_2$ -resistant subline. *Proc Jap Cancer Ass*, 21st General Meeting, 144-145, 1962.
- (15) KOLLER, P. C.: Chromosome behavior in tumors: Readjustment to Boveri's theory. *Cell Physiol Neoplas*, Univ Texas Press, 1960, 10-37.
- (16) LEVAN, A., and HAUSCHKA, T. S.: Endomitotic reduplication mechanisms in ascites tumors of the mouse. *J Nat Cancer Inst* 14: 1-43, 1953.
- (17) MAKINO, S.: The chromosome cytology of the ascites tumors of rats, with special reference to the concept of the stem cell. *Int Rev Cytol* 6: 25-84, 1957.
- (18) MAKINO, S., and SASAKI, M.: Cytological studies of tumors. XXI. A comparative ideogram study of the Yoshida sarcoma and its subline derivatives. *J Nat Cancer Inst* 20: 465-487, 1958.
- (19) NAKAMURA, K.: Studies on the ascites hepatoma. VIII. Difference of chromosome numbers among tumor cells constituting the same "island" of the ascites hepatoma. *Gann* 46: 196-199, 1955.
- (20) NAKAMURA, K., ODASHIMA, S., ISAKA, H., and KURATA, T.: Studies on the ascites hepatoma. X. Further studies on the chromosome number. *Gann* 47: 502-504, 1956.
- (21) ODASHIMA, S.: Studies on the ascites hepatoma. XXI. Ascitic conversion of the liver cancers developed in rat fed with 3'-Me-4-DAB. *Proc Jap Cancer Ass*, 20th General Meeting, 91, 1961.
- (22) SATO, H.: Staining of tumor cells by Acetogentian-violet-solution. *Trans Soc Path Jap* 40: 139-140, 1951.
- (23) ———: On the chromosomes of Yoshida sarcoma. Studies with tumor cells proliferated in the peritoneal cavity of the rat transplanted with a single cell. *Gann* 43: 1-16, 1952.
- (24) SATO, H., and ATSUMI, A.: On the multinucleate cells in the Yoshida sarcoma. *Gann* 43: 300-301, 1952.
- (25) SATO, H., ESSNER, E., and BELKIN, M.: Experiments on an ascites hepatoma. II. Intraperitoneal transplantation of free tumor cells separated from islands of the rat ascites hepatoma. *Exp Cell Res* 9: 381-392, 1955.
- (26) SATO, H., ATSUMI, S., SATOH, H., and NAKAMURA, K.: Time necessary for the completion of a mitosis in the Yoshida sarcoma cell (mathematically estimated value and actually measured value). *Gann* 43: 303-306, 1952.
- (27) TANAKA, T., and KANO, K.: On the somatic chromosomes of rats. *Cytologia Suppl Int Genetics Sympos Tokyo*, 196-201, 1957.

- (28) TJIO, J. H., and LEVAN, A.: Chromosome analysis of three hyperdiploid ascites tumors of the mouse. *K Fysiogr Sällsk Handl, N. F.* 65: 1-39, 1954.
- (29) ———: Comparative idiogram analysis of the rat and the Yoshida rat sarcoma. *Hereditas* 42: 218-234, 1956.
- (30) YOSHIDA, T.: Contribution of the ascites hepatoma to the concept of malignancy of cancer. *Ann NY Acad Sci* 63: 852-881, 1956.
- (31) ———: Studien über das Ascites-Hepatom. Zugleich ein Beitrag zum Begriff der cellulären Autonomie im Wachstum der malignen Geschwulst einerseits, und der Individualität der einzelnen Geschwulst anderseits. *Virchow Arch Path Anat* 330: 83-105, 1957.
- (32) ———: Screening with ascites hepatoma. *Ann NY Acad Sci* 76: 610-618, 1959.
- (33) ———: On the ascites hepatoma. Summary of the results of studies obtained during 10 years from 1951 to 1960. *Tokyo J Med Sci* 68: 717-748, 1960. *Sci* 68: 717-748, 1960.
- (34) YOSHIDA, T., ISAKA, H., ODASHIMA, S., ISHIDATE, M., JR., and YAMADA, T.: Studies on the ascites hepatoma. XIX. Further studies on the chromosome. *Proc Jap Cancer Ass, 19th Meeting*, 88, 1961.
- (35) YOSHIDA, T., SATO, H., and ATSUMI, A.: On refusion of once completely separated daughter cells. A contribution to the genesis of binucleate cell by phasemicroscopic observation of Yoshida sarcoma cells. *Proc Jap Acad* 26: 48-54, 1950.
- (36) YOSIDA, T. H.: Origin of V-shaped chromosome occurring in tumor cells of some ascites sarcomas in the rat. *Proc Jap Acad* 31: 237-242, 1955.

PLATES

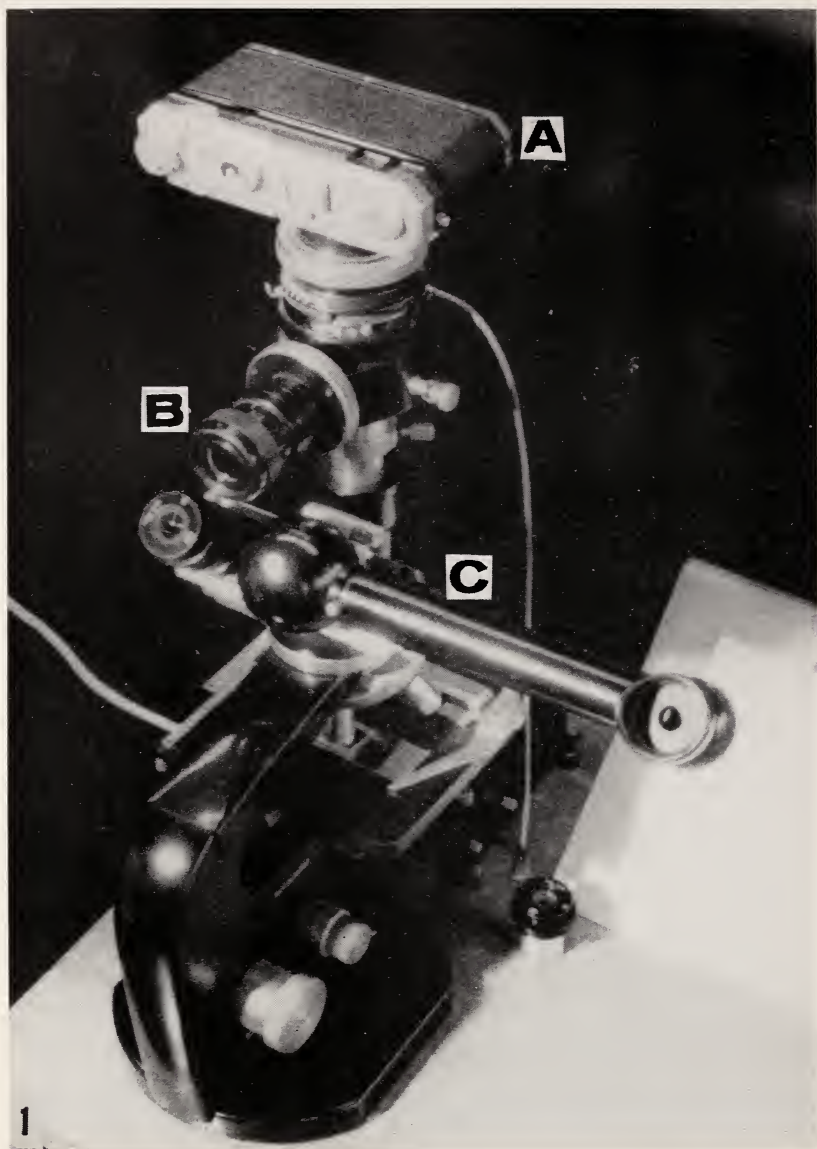
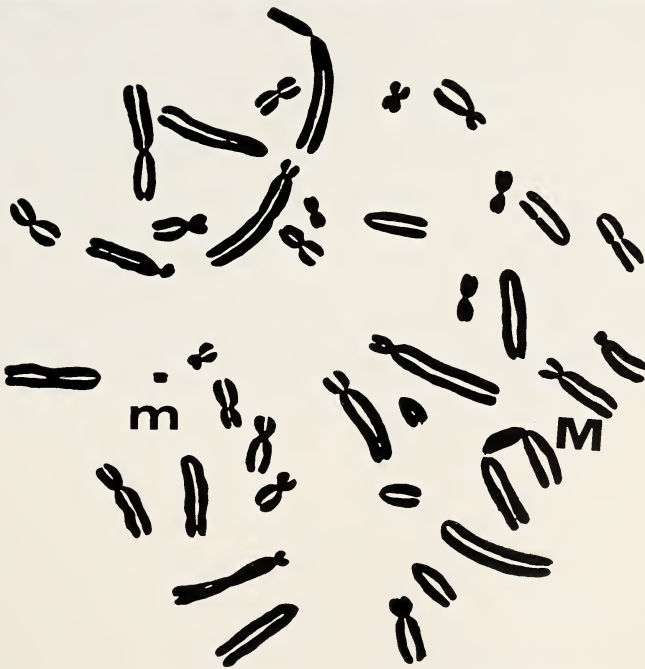
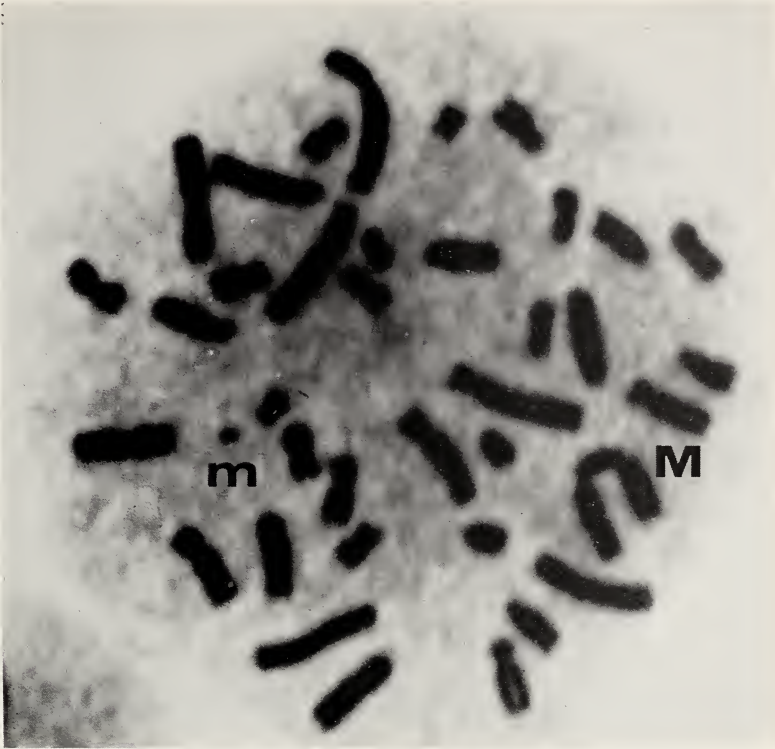


FIGURE 1.—Apparatus for chromosome examinations: 35 mm camera (A), lens for observation of nuclear plates at the time of photography (B), and projection apparatus (C) attached to the binocular microscope.



2

FIGURE 2.—An example of the photomicrograph (*top*) and drawing (*bottom*) of a metaphase plate of AH 66F cell.

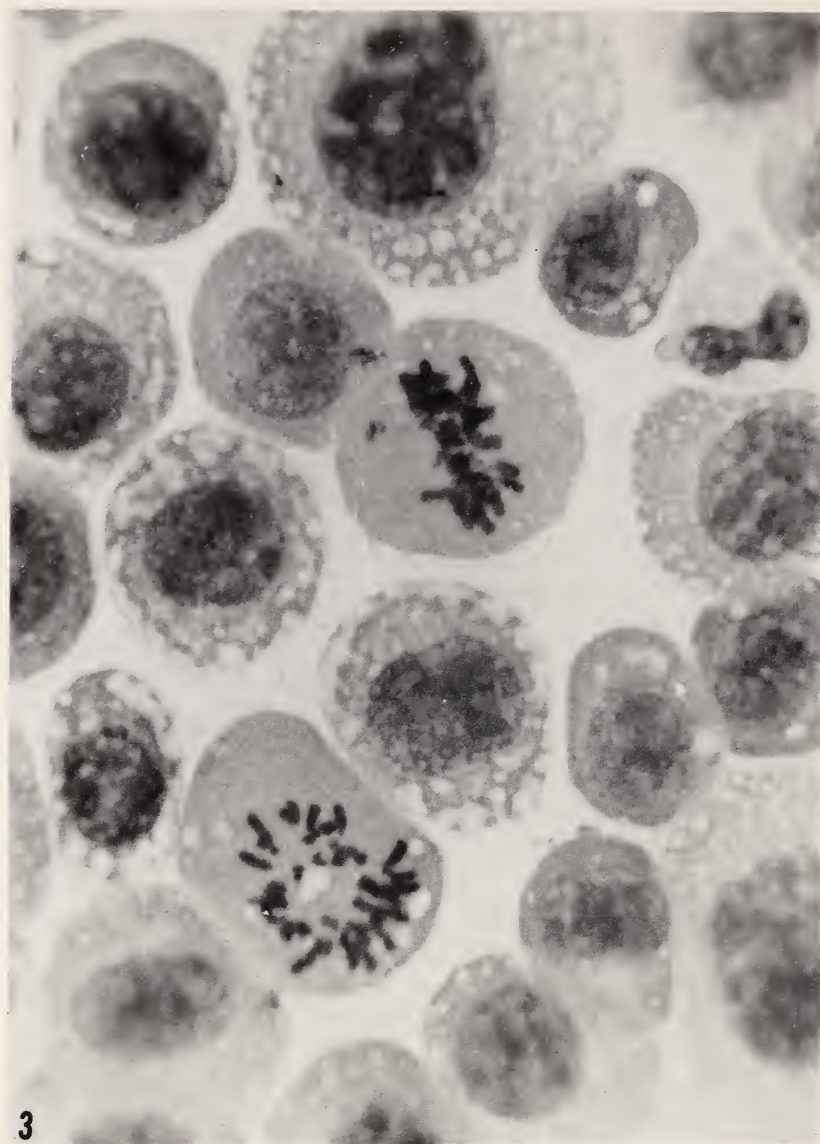


FIGURE 3.—Ascites hepatoma AH 66F, showing abundant free tumor cells.

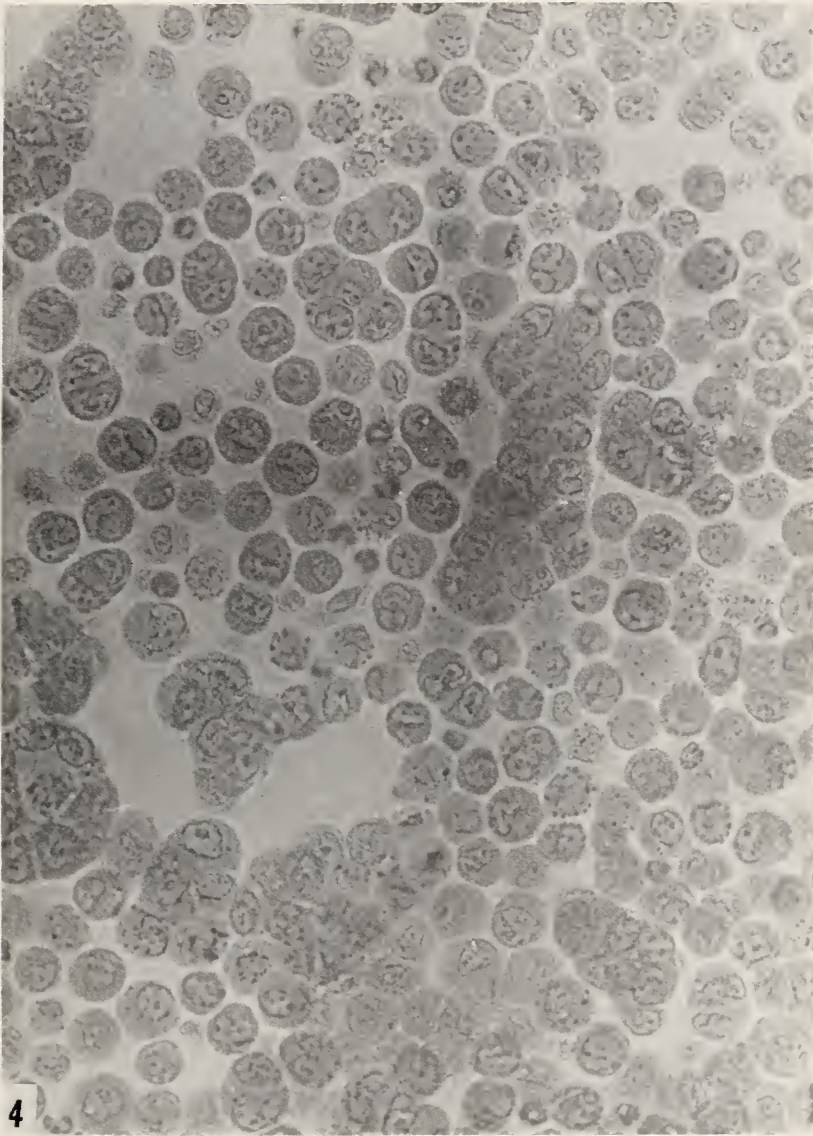
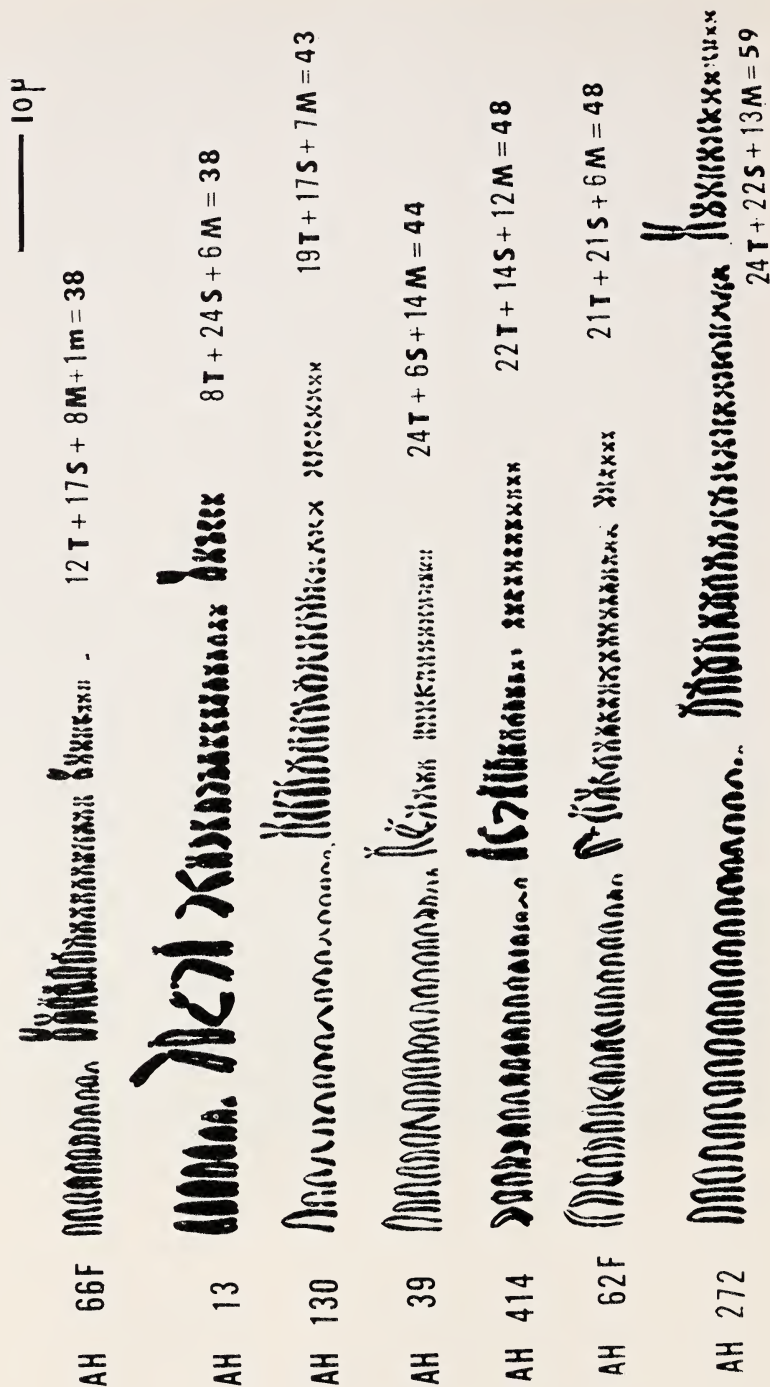


FIGURE 4.—Ascites hepatoma AH 130, showing abundant free tumor cells and small islands.



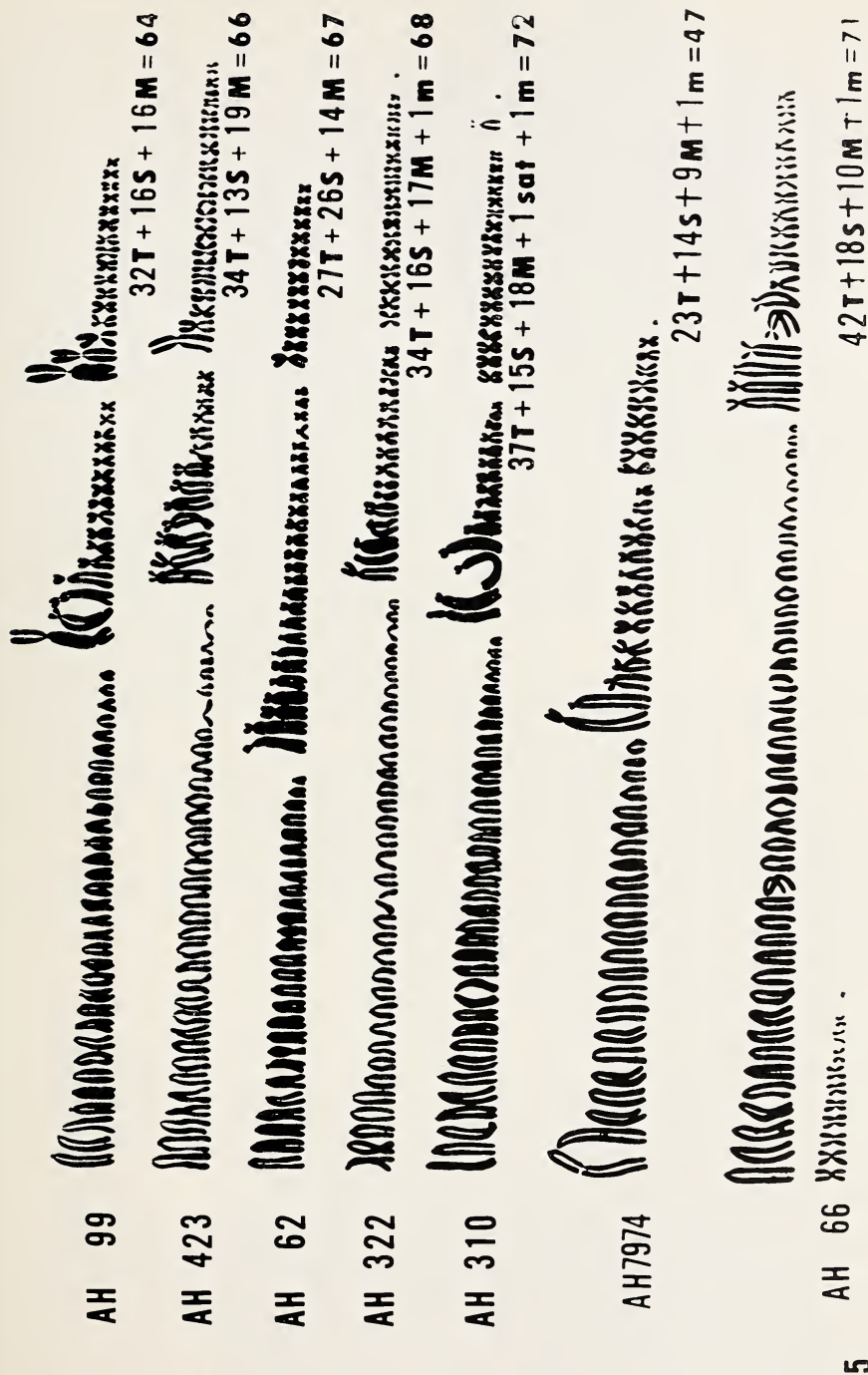


FIGURE 5.—Chromosome idiograms of ascites hepatoma cells: strains AH 66F, AH 13, AH 130, AH 39, AH 414, AH 62F, AH 272, AH 99, AH 423, AH 62, AH 322, AH 310, AH 7974, and AH 66.

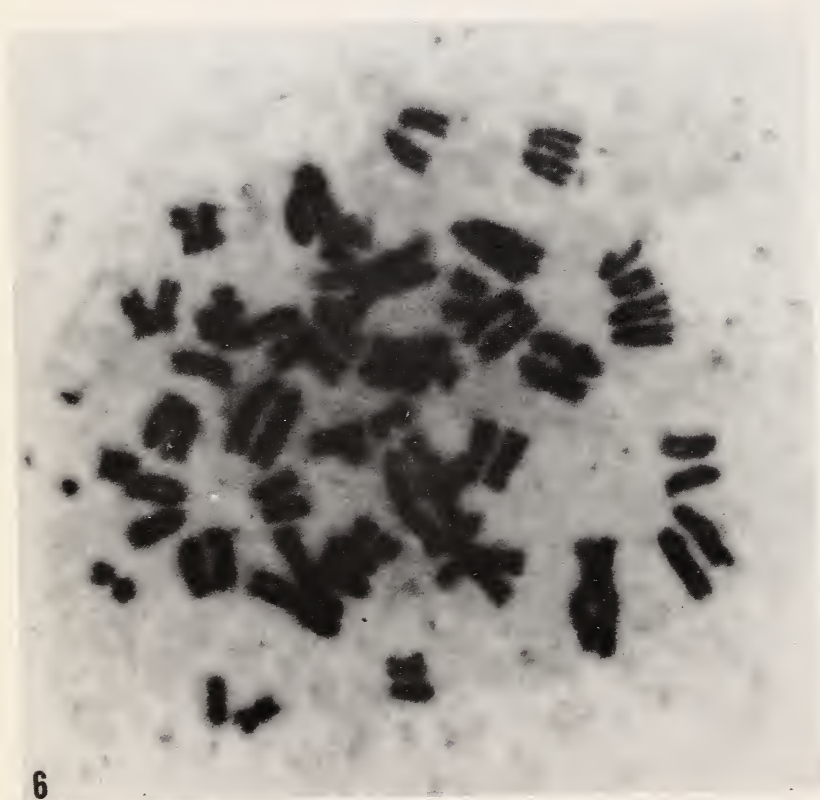


FIGURE 6.—A nuclear plate of endomitotic reduplication. AH 39 cell.

FIGURE 7→  
Three-celled island.  
Chromosome  
idiograms of cells  
belonging to island  
AH 130.

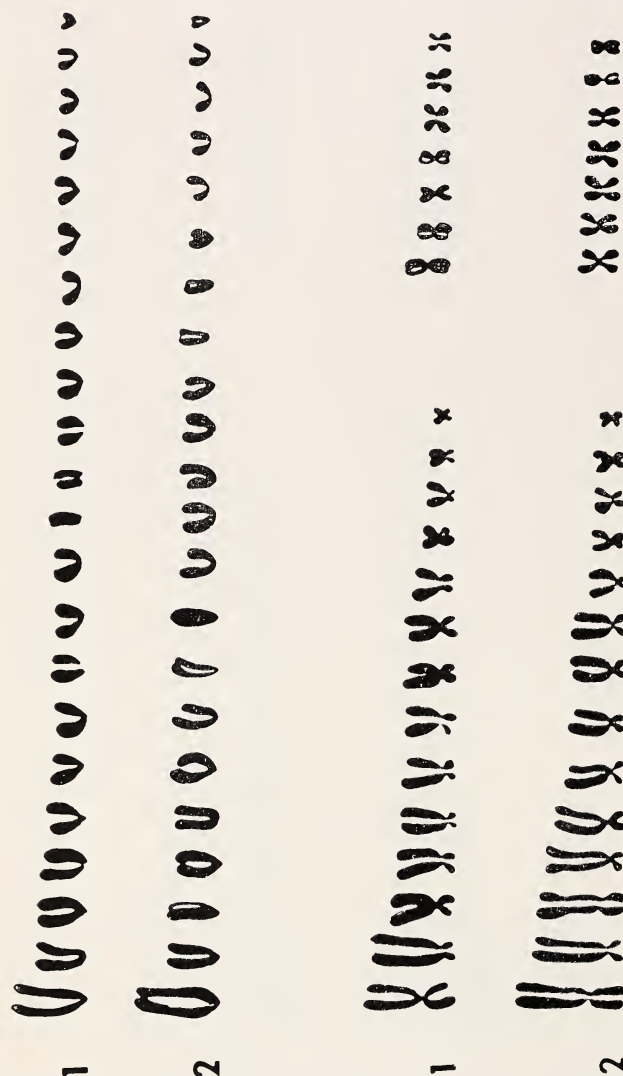




FIGURE 8.—Eight-celled island. Chromosome idiograms of cells belonging to island AH 130.



FIGURE 9.—Nine-celled island. Chromosome idiograms of cells belonging to island AH 130.



Island No. 1 ; 11-celled

Chromosomes ; 1 :  $122 + S14 + M7 = 43$   
 2 :  $122 + S14 + M7 = 43$

10

Figure 10.—Eleven-celled island. Chromosome idiograms of cells belonging to island AH 130.

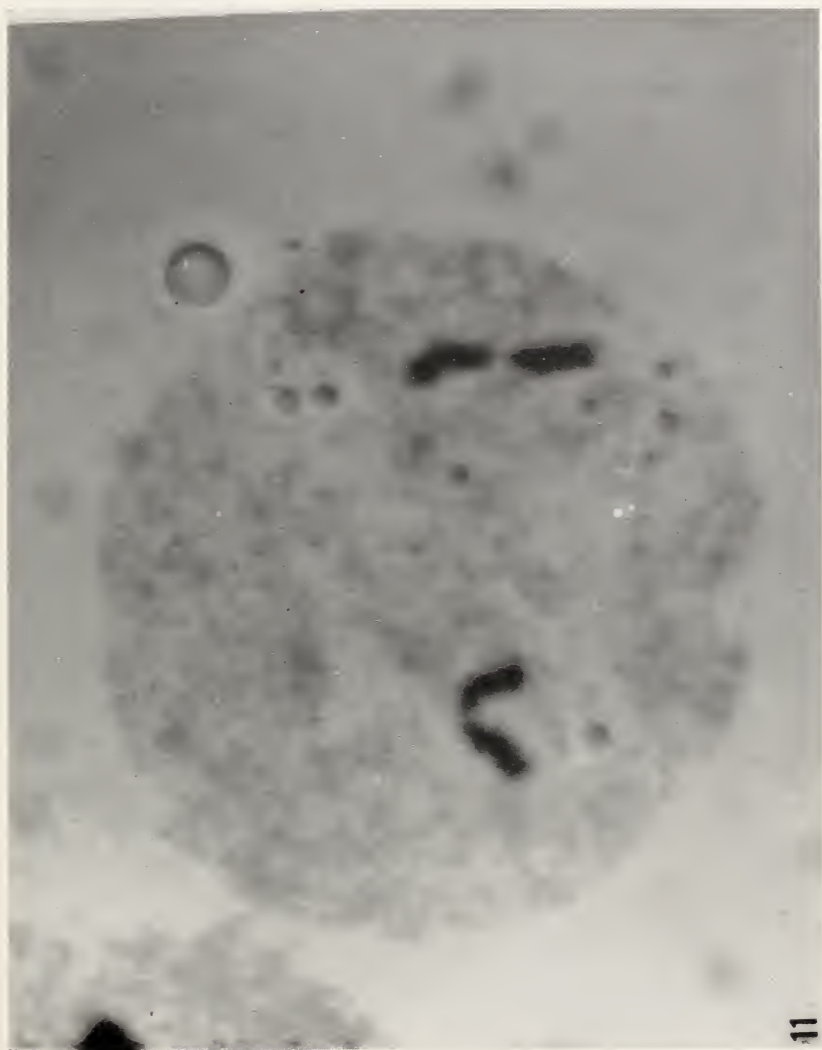


FIGURE 11.—Chromosome #2. A11 39. Ascites hepatoma cells with smaller number of chromosomes.

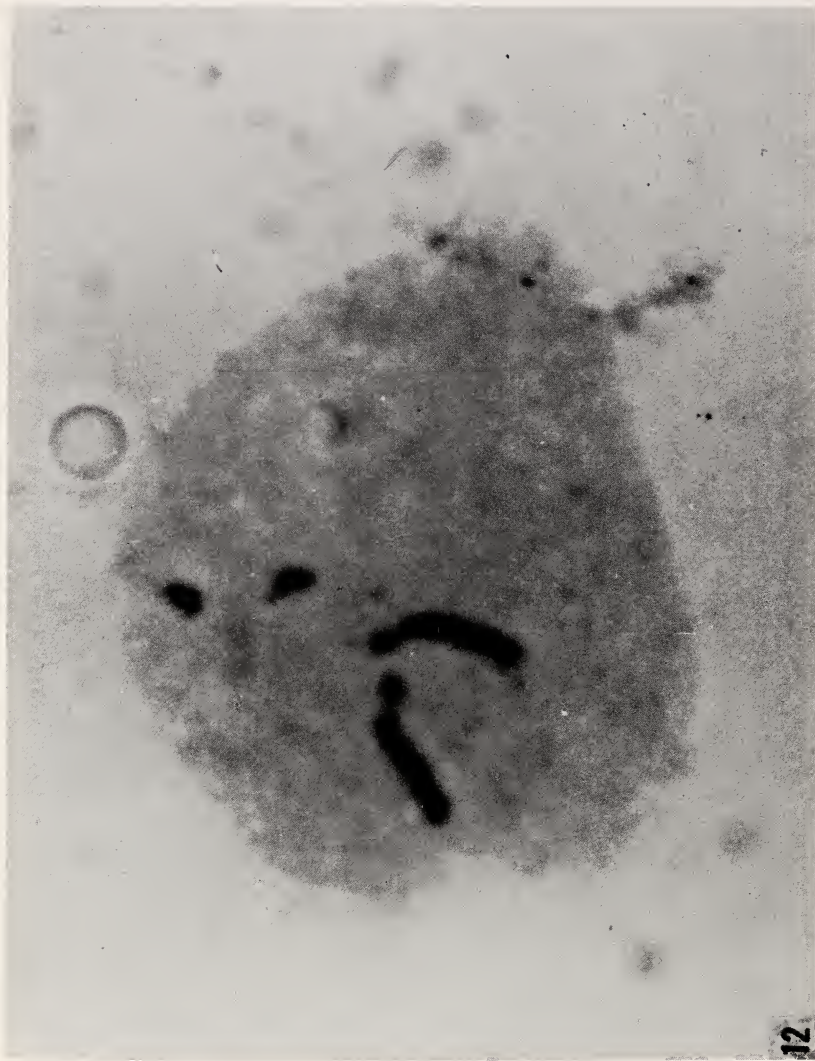


FIGURE 12.—Chromosome #2. AH 39. Ascites hepatoma cells with smaller number of chromosomes.

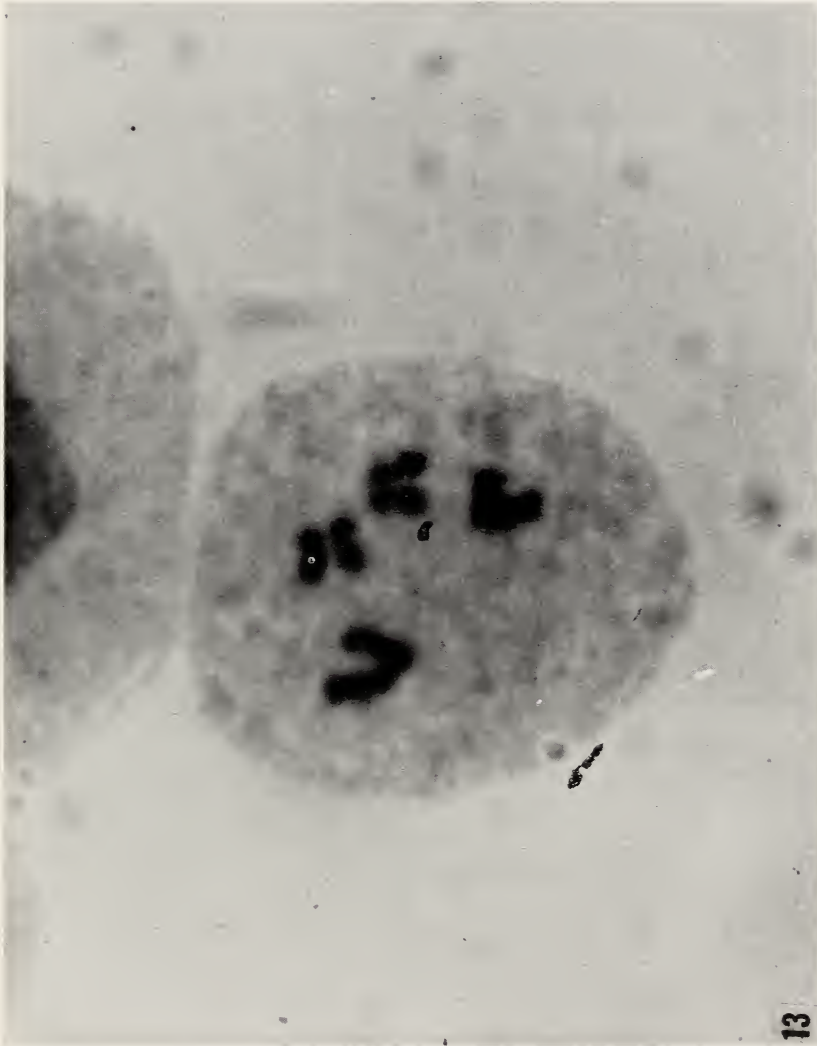


Figure 13.—Chromosome #4. AH 39. Ascites hepatoma cells with smaller number of chromosomes.

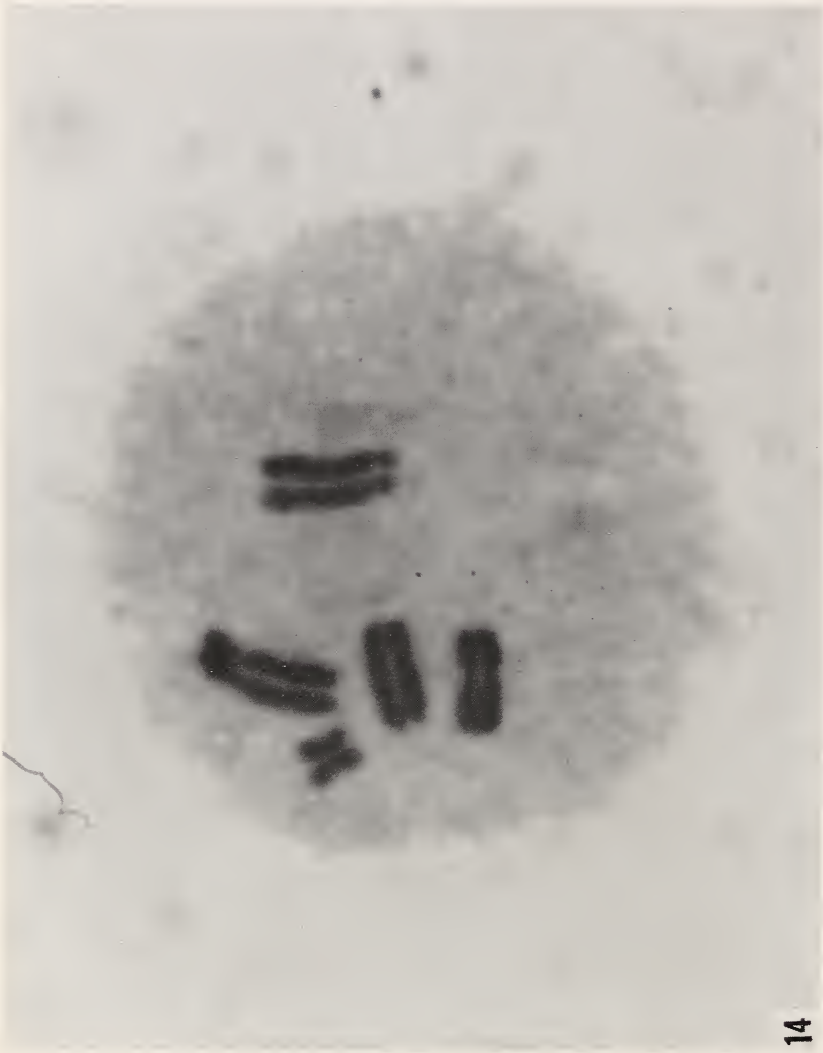


Figure 14.—Chromosome #5. All 13. Ascites hepatoma cells with smaller number of chromosomes.

## **“Natural” Drug Resistance of the Ascites Hepatoma in the Rat<sup>1</sup>**

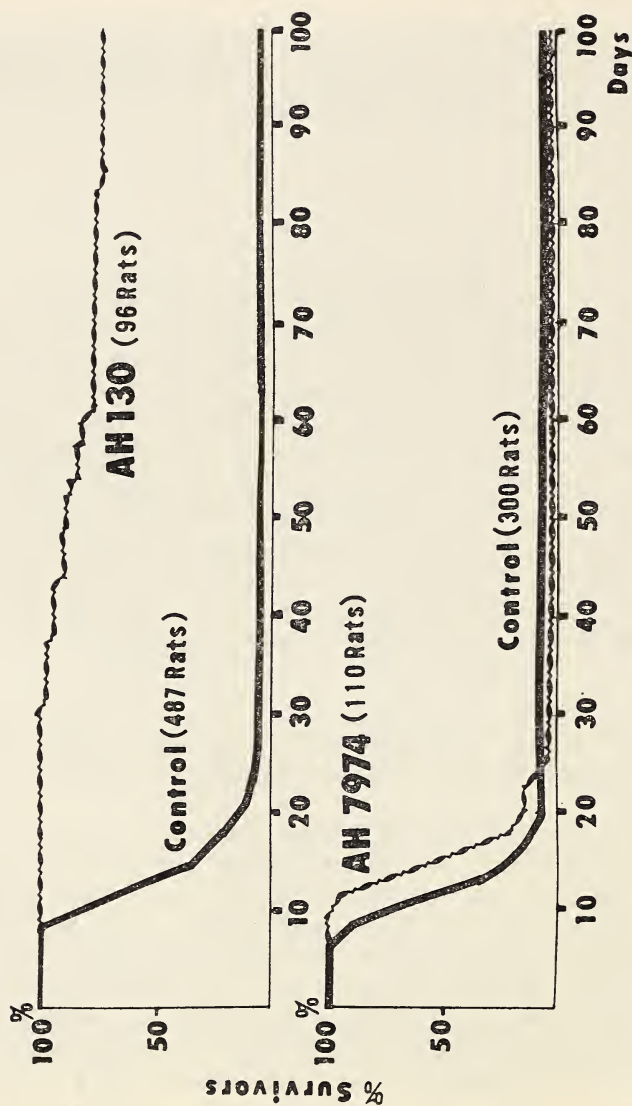
HIDEHIKO ISAKA, *Sasaki Institute, Sasaki Foundation, Tokyo, Japan*

**M**ALIGNANT hepatomas induced in rats fed amino azo compounds such as 4-*o*-tolylazo-*o*-toluidine and *N, N*-dimethyl-*p*-phenylazoaniline were converted to the ascites form. Since 1951 we have successfully established 57 different transplant strains of ascites hepatomas in hybrid Japanese albino or “Donryu” rats, a highly susceptible, closed colony of our own breed, and maintained them by serial animal passages. Thus we have a variety of ascites hepatomas for experimental studies. We compared the following characteristics of these different tumor strains: 1) growth rate, 2) transplantability, 3) variations in ascites, 4) ability to metastasize, and 5) chromosome constitution. The results obtained so far indicate that each strain of ascites hepatoma has its own individual characteristics (1, 2).

### **“NATURAL” RESISTANCE OF THE ASCITES HEPATOMA TO NITROGEN MUSTARD N-OXIDE (NITROMIN)**

The most striking difference in the ascites hepatomas was the variation in their response to chemotherapeutic agents. Satoh (3) first observed that the ascites hepatoma AH 7974 had always been resistant to Nitromin but that strain AH 130 responded to the same drug in more than 80 percent of the animals treated (text-fig. 1). Further examination of 25 strains of ascites hepatomas showed that 18 of 25 tumors were refractory, while the remaining 7 responded favorably to the compound. With 30 different compounds, including TEM, Myleran, thioTEPA, Degranol, Endoxan, and RC-4, similar reactions were clearly demonstrated (1), *i.e.*, the ascites hepatoma strains that responded well to Nitromin were also sensitive to other alkylating agents. Cytological examination of the ascitic fluid from treated animals, bearing sensitive strains of these hepatomas, showed that there were marked cellular changes which sug-

<sup>1</sup> Supported in part by grant CY-2799 from the National Cancer Institute, National Institutes of Health, Public Health Service, and contract DA-92-557-FEC-35765, U.S. Army, Far East Research Office.



TEXT-FIGURE 1.—Difference of effects of Nitrovin on ascites hepatomas AH 130 and AH 7974.

gested an eventual "cure" or marked prolongation of survival time of the host.

One criterion used for evaluating the antineoplastic potency of a chemotherapeutic agent, both in humans and laboratory animals, is reduction in size of all measurable tumor nodules. Other criteria used for the ascites tumors include the volume of ascites and survival time of the treated host. If the tumor cells are highly sensitive to the drug there may be a "cure" or prolongation of survival time of the host. With a low sensitivity, a dose necessary to affect the tumor cells will surpass the tolerance dose of the host. Evidence obtained from additional experimental studies suggests that other criteria should be considered. One factor that may determine the response of tumors to chemotherapeutic agents is an inherent property of the tumor cell itself.

Satoh (3) demonstrated the sensitivity of 55 different stock lines of ascites hepatomas to Nitromin by evaluating sensitivity according to the morphological changes that occurred in the tumor cells. Yoshida (4) reported that nitrogen mustard and its derivative compounds induce a characteristic cytological effect on ascites tumor cells and this is referred to as the "HN<sub>2</sub> effect." Some of the effects produced by the drug are dispersion, clumping, chromosome breaks, chromosome bridges, and the formation of giant cells with necrosis. All these marked changes in the cells may be used as indicators of sensitivity, and the more changes that occur the greater the inhibitory effect on the tumor.

In our study ascites hepatoma cells were treated with Nitromin *in vivo* or *in vitro* and examined cytologically. The amount of the drug required to induce an effect on half the tumor cells was defined as the "minimum effective dose" (MED).

#### *In Vivo* Test

The rats received single intraperitoneal injections of various dosages of Nitromin 4 days after transplantation of the ascites hepatomas. The ascitic fluid was examined cytologically. Smears were made and stained with Wright-Giemsa at 24-hour intervals for 4 days after injection of the drug. This procedure was repeated, and the minimum amount of the drug necessary to induce the HN<sub>2</sub> effect on half the tumor cells was considered as the MED *in vivo*.

#### *In Vitro-In Vivo* Test (1)

One ml of a 4-day-old ascites hepatoma, containing about 10<sup>8</sup> tumor cells, was mixed in a test tube with Nitromin dissolved in 1 ml of physiological saline, incubated for 30 minutes at 37° C, and then transplanted into the peritoneal cavity of a normal rat. When ascites developed smears were made and stained with Wright-Giemsa at 24-hour intervals for 4 days and examined microscopically. The minimum dose of the drug required to induce the cytological effect was thus determined as the MED *in vitro-in vivo*. The results are shown in table 1.

TABLE 1.—Minimum effective dose (MED) *in vivo* and *in vitro-in vivo* and the maximum tolerance dose (MTD) of Nitromin in 55 ascites hepatomas

Strains	MED <i>in vivo</i> (mg/kg)	MED <i>in vitro-in vivo</i> ( $\mu$ g/ml)	MTD (mg/kg)
AH 122A	> 50.0	—	50.0
AH 122B	> 50.0	—	50.0
AH 311	> 50.0	—	50.0
AH 42B	50.0	—	50.0
AH 44	50.0	—	50.0
AH 70B	50.0	—	50.0
AH 109A	50.0	—	50.0
AH 143A	50.0	—	50.0
AH 210A	50.0	—	50.0
AH 149	50.0	50.0	50.0
AH 286	50.0	50.0	50.0
AH 322	50.0	50.0	50.0
AH 66	50.0	10.0	50.0
AH 408	50.0	10.0	50.0
AH 423	50.0	10.0	50.0
AH 7974	50.0	10.0	50.0
AH 173	50.0	5.0	50.0
AH 41A	25.0	—	50.0
AH 106B	25.0	—	50.0
AH 107B	25.0	—	50.0
AH 131A	25.0	—	50.0
AH 131B	25.0	—	50.0
AH 310	25.0	10.0	50.0
AH 127	25.0	5.0	50.0
AH 62	20.0	10.0	50.0
AH 62F	20.0	10.0	50.0
AH 41B	10.0	—	50.0
AH 34	10.0	—	50.0
AH 61B	10.0	—	50.0
AH 136B	10.0	—	50.0
AH 371A	10.0	—	50.0
AH 49	10.0	10.0	50.0
AH 318	10.0	10.0	50.0
AH 21	10.0	5.0	50.0
AH 63	10.0	5.0	50.0
AH 602	10.0	0.5	50.0
AH 39	7.5	1.0	50.0
AH 41C	5.0	—	50.0
AH 57B	5.0	—	50.0
AH 60C	5.0	—	50.0
AH 84A	5.0	—	50.0
AH 84B	5.0	—	50.0
AH 108A	5.0	—	50.0
AH 150A	5.0	—	50.0
AH 255A	5.0	—	50.0
AH 7974F	5.0	—	50.0
AH 414	5.0	5.0	50.0
AH 55A	1.0	—	50.0
AH 65C	1.0	—	50.0
AH 272	1.0	0.5	50.0
AH 601	1.0	0.5	50.0
AH 13	1.0	0.1	50.0
AH 66F	1.0	0.1	50.0
AH 99	1.0	0.1	50.0
AH 130	1.0	0.1	50.0

A marked variation in sensitivity to the drug is evident. The MED for strains AH 13, AH 66F, AH 99, and AH 130 was low, while that for AH 149, AH 286, and AH 322 was very high. The MED *in vivo* for strains AH 122A, AH 122B, and AH 311 could not be determined exactly because the MED *in vivo* was probably so high that it exceeded the maximum tolerance dose (MTD) of the drug on the host. The MED of the other strains varied in sensitivity (table 1). The MED's *in vivo*, for the ascites hepatomas listed in table 1, did not always show correlation with their MED's *in vitro-in vivo*. This might suggest that variation in sensitivity of the tumor to chemotherapeutic drugs may be related to a "natural" drug resistance. This "natural" resistance is used here to denote an inherent property characteristic of each tumor strain, in contrast to "acquired" resistance which develops following repeated contact of the tumor cells with the drug.

Irako (5) determined the lethal dose *in vitro* of X rays on 20 ascites hepatoma strains. Table 2 shows the lethal dose, *i.e.*, the minimum X-ray dose necessary to kill  $10^7$  cells *in vitro* for these strains of ascites tumors. He found that different strains had different degrees of radio-sensitivity. However, there was no significant correlation between resistance to X irradiation and Nitromin, *i.e.*, not all strains that are highly resistant to X irradiation have high resistance to Nitromin and vice versa.

In considering natural resistance to alkylating agents and X ray, a "spectrum" of the ascites hepatoma might be established. It would be of interest to determine whether a similar spectrum could be extended to other series of cancer chemotherapeutic drugs, such as antibiotics and metabolic antagonists.

TABLE 2.—Lethal X-ray dose *in vitro* on 20 ascites hepatomas

Strains	Lethal X-ray dose <i>in vitro</i> on $10^7$ tumor cells (r)
AH 7974	5,000
AH 13	5,000
AH 66F	5,000
AH 99	5,000
AH 21	4,000
AH 318	4,000
AH 364	4,000
AH 408	4,000
AH 62	3,000
AH 63	3,000
AH 49	3,000
AH 66	3,000
AH 130	3,000
AH 322	3,000
AH 414	3,000
AH 423	3,000
AH 602	3,000
AH 39	2,000
AH 149	2,000
AH 602	2,000

# POPULATION ANALYSIS OF ASCITES HEPATOMAS AND "NATURAL" DRUG RESISTANCE TO NITROMIN

For many years it has been stated that a tumor is not homogeneous but is composed of a mosaic complex of cells with different characteristics. This "mosaic concept" was supported by various studies including transplantability, growth rate, and chromosome constitution (6-9). We have also demonstrated that the tumor cell population consists of cells with various chromosome numbers and that stable polyploids as well as diploids can develop from single cells such as the ascites hepatoma AH 66F and the Yoshida ascites sarcoma (10, 11). After establishing many clonal sublines from the stock strains, we studied the population analysis of ascites hepatomas to determine whether a mosaic population of neoplastic cells affects the natural drug resistance of certain tumors (12, 13).

Strains AH 272, AH 414, and AH 39 that contain many individual isolated cells in ascites form, as shown in figures 1, 2, and 3, were used. Single cell transplantation was utilized to establish clonal tumor cell population (see "Chromosome Studies of Various Strains of Ascites Hepatomas in Rats" in this issue). The MED of the cells from the clonal tumors and from their original population was compared.

Of 60 rats that received a single cell transplant of strain AH 272, 31 developed ascites, and the MED *in vivo* of 25 clonal populations of these 31 clones was successfully determined. Twelve clonal populations resulted from 100 single cell transplantations of strain AH 414, and the MED of 10 clones determined (tables 3 and 4). From a single original transplanted cell of either strain AH 272 or AH 414, 3 groups of clones were derived which differed in their MED *in vivo* to Nitromin. One clone had the same MED as the parent line of the tumor, but the MED of the remaining 2 clones was higher or lower than the parent line. This means that the original population of AH 272 or AH 414 is not homogeneous but a mosaic composition of at least 3 types of tumor cells differing from each other in their natural resistance to Nitromin. The degree of resistance of the original tumor cell population, therefore, is the average of all of its constituents which differ in their degree of resistance.

TABLE 3.—Resistance of clonal sublines of ascites hepatoma AH 272 to Nitromin

Number of clones examined	MED* <i>in vivo</i> (mg/kg)
6	2.5
17	1.0
2	0.5
AH 272, parent line	1.0

\*MED in this and the following tables denotes the minimum effective dose.

TABLE 4.—Resistance of clonal sublines of ascites hepatoma AH 414 to Nitromin

Number of clones examined	MED <i>in vivo</i> (mg/kg)
3	10.0
5	5.0
2	2.5
AH 414, parent line	5.0

A study of strain AH 39 revealed that the population of neoplastic cells varied in their degree of drug resistance.

Table 5 shows the MED *in vivo* of 11 clones that resulted from 95 single cell transplantations of AH 39 whose original population was a mosaic of cells with different degrees of drug resistance: The MED of the parent line was 7.5, whereas the MED of clones derived from this line varied from 1 to 50. It can be seen that the parent line of AH 39 contains cells showing the high degree of resistance of 50—the same as the maximum tolerance dose of the host.

TABLE 5.—Resistance of clonal sublines of ascites hepatoma AH 39 to Nitromin

Number of clones examined	MED <i>in vivo</i> (mg/kg)
1	50.0
1	25.0
2	10.0
4	7.5
2	5.0
1	1.0
AH 39, parent line	7.5

Table 6 shows the MED *in vivo* of 4 different clones of AH 39 during their serial animal passages. The highly resistant clones decreased gradually, while those of lower resistance increased throughout their passages. In general, the degree of resistance fluctuated at about 7.5, the MED of the original population. This fluctuation was evidently limited within the MED levels of 1 to 50 of the different clones of the parent line. This observation may imply that the degree of natural drug resistance of a whole tumor, as well as its constituents, is not a definitely fixed value but can fluctuate naturally within a limit corresponding to the range of variations of cells in the population. It is significant that the resistance of the original stock population of AH 39 has fluctuated within a relatively narrow range of 5 to 10 MED during the prolonged period of transplantation.

TABLE 6.—Resistance to Nitromin of clonal sublines of AH 39 during serial animal passages

Clone No.	Generation	MED <i>in vivo</i> (mg/kg)				
		1st	5th	8th	20th	30th
1		50	25	25	5	5
2		25	7.5	5	5	1
3		7.5	7.5	7.5		
4		5	7.5	10	25	5

## DRUG RESISTANCE OF TUMOR CELL POPULATIONS OF ASCITES HEPATOMA CELLS AFTER FREEZING

Isaka was the first to observe that the degree of drug resistance of Yoshida sarcoma could be varied by storage at  $-80^{\circ}\text{C}$  in the frozen tumor bank (14). Twenty-five different strains of ascites hepatomas kept at  $-80^{\circ}\text{C}$  in the frozen tumor bank, for periods ranging from 4 to 13 months, were thawed and injected into normal rats and the tumor cell populations that developed were examined for their MED's. The procedure was as follows: A nearly pure culture of tumor ascitic fluid was mixed well with pure glycerol. The volume ratio of glycerol to ascitic fluid was 1:9. The mixture was sealed in a glass ampoule and immediately placed in a  $-80^{\circ}\text{C}$  deepfreeze. After varying periods of storage, the ampoule was removed from the freezer and instantly placed in a water bath at  $37^{\circ}\text{C}$  and shaken for 2 minutes. The thawed ascites tumor, 0.2 ml, was injected intraperitoneally into normal rats. The results are shown in table 7. In 14 of 25 strains examined, the MED of Nitromin in 13 strains was greater after freezing and in the remaining 1 strain was lower than the MED of the original tumors.

TABLE 7.—Resistance of ascites hepatomas to Nitromin before and after freezing at  $-80^{\circ}\text{C}$  for 4 to 13 months \*

Strains	MED <i>in vivo</i> (mg/kg)	
	Before	After
AH 7974	50.0	>50.0
AH 66	50.0	>50.0
AH 423	50.0	>50.0
AH 127	25.0	50.0
AH 49	10.0	50.0
AH 602	10.0	50.0
AH 318	10.0	25.0
AH 63	10.0	25.0
AH 39	7.5	50.0
AH 414	5.0	25.0
AH 601	1.0	10.0
AH 66F	1.0	5.0
AH 272	1.0	2.5
AH 13	1.0	0.5

\* In 11 other strains no difference was observed.

With the tumor ascites of strain AH 39, the same experiment was repeated 38 times. The cold-storage period was 48 hours throughout the experiment. Table 8 shows the MED values that ranged from 5 to 50, which was within the natural range of variation in the established clones of AH 39. The tumors developing higher degrees of resistance after cold storage showed about the same MED value of the original tumor AH 39 when further transplanted.

TABLE 8.—Resistance to Nitromin of ascites hepatoma AH 39 after freezing at  $-80^{\circ}\text{C}$  for 48 hours

Number of specimens examined	MED <i>in vivo</i> (mg/kg)
2	50.0
5	25.0
18	10.0
11	7.5
2	5.0
AH 39, control	7.5

The heterogeneous population of cancer cells observed may be explained in various ways. It is generally accepted that each cell of a tumor has its own degree of stable natural drug resistance, and all descendants of one cell, except rare mutants, maintain this same degree of resistance. However, the observed phenomenon of fluctuation in resistance that has been described can be explained as follows: Cells of a neoplastic population, even the descendants in a clonal population, can vary in their natural drug resistance within a certain limited range that is probably specific to each tumor. The cancer cell will show its variability as long as it proliferates. Thus, the fluctuation in drug resistance of a tumor may be a matter of pronounced natural variation. In this connection, it may be noted that diploid as well as polyploid stable clones isolated from the Yoshida sarcoma have shown the same resistance to  $\text{HN}_2$  (11).

Thus, acquired drug resistance, observed with malignant tumors, may be caused by a certain number of neoplastic cells that develop a drug resistance which exceeds the range of natural drug resistance.

## REFERENCES

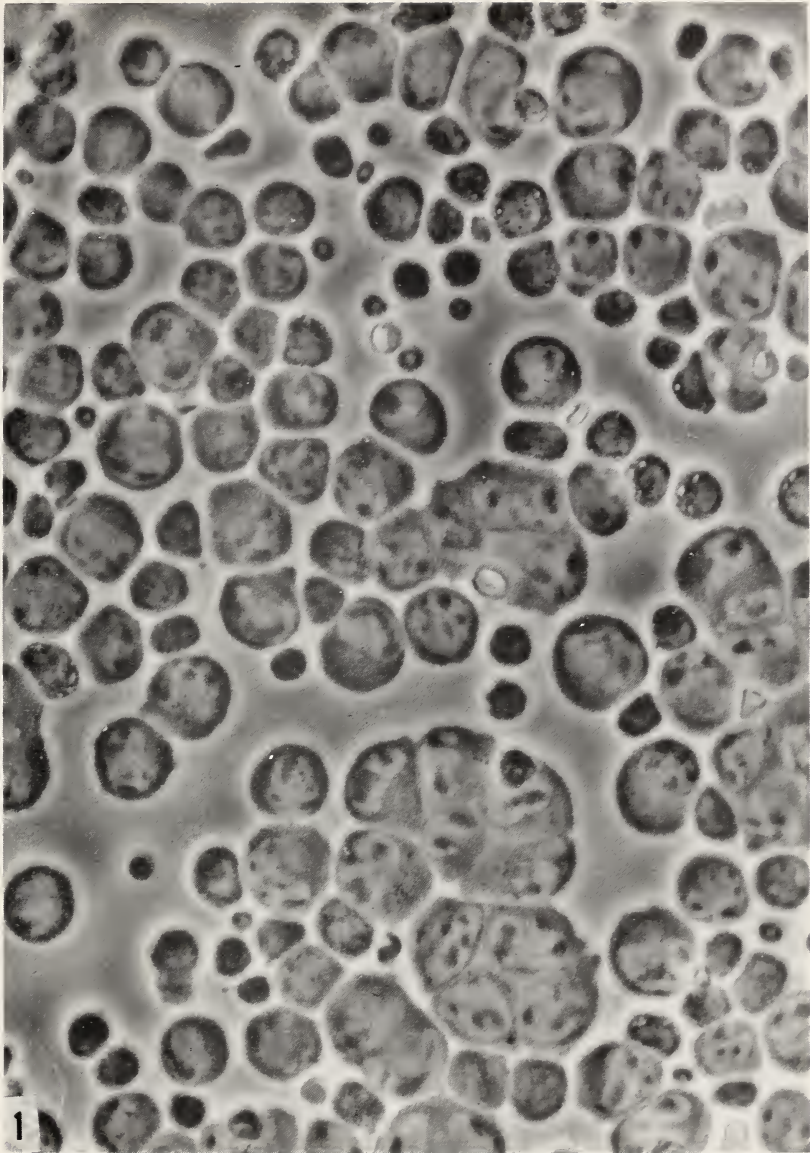
- (1) YOSHIDA, T.: Screening with ascites hepatoma. *Ann NY Acad Sci* 76: 610-618 1958.
- (2) ———: On the ascites hepatoma. Summary of the results of studies obtained during 10 years from 1951 to 1960. *Tokyo J Med Sci* 68: 717-748, 1960.
- (3) SATOH, H: Studies on the ascites hepatoma. XI. Different responses by different strain of ascites hepatomas of rats to chemotherapeutic treatment. *Gann* 47: 334-337, 1956.
- (4) YOSHIDA, T.: Studies on an ascites (reticuloendothelial cell ?) sarcoma of the rat. *J Nat Cancer Inst* 12: 947-969, 1952.

- (5) IRAKO, Y.: Effect of X-radiation on the ascites tumors: difference in radio-sensitivity among various transplant-strains of the ascites hepatoma of the rat. *Gann* 51: 33-45, 1960.
- (6) HAUSCHKA, T. S.: Methods of conditioning the graft in tumor transplantation. *J Nat Cancer Inst* 14: 723-739, 1953.
- (7) ———: Tissue genetics of neoplastic populations. Canadian Cancer Conference. New York, Academic Press Inc., 1957, vol. 2, pp 305-345.
- (8) ———: Correlation of chromosomal and physiologic changes in tumors. *J Cell Comp Physiol* 52 (Suppl 1): 197-233, 1958.
- (9) KLEIN, G.: The usefulness and limitation of tumor transplantation in cancer research: a review. *Cancer Res* 19: 343-358, 1959.
- (10) ISAKA, H.: On the chromosomes of ascites tumors. Presented at a symposium on Characteristics of Cancer Growth at a special autumn meeting of the Japanese Pathological Society, 1962.
- (11) ISAKA, H., SATOH, H., and OISHI, Y.: Studies on the chromosomes of two different sublines of Yoshida ascites sarcoma; a polyploid subline and an  $HN_2$ -resistant subline. *Proceedings of Japanese Cancer Association, 21st General Meeting, 1962*, pp 144-145.
- (12) ISAKA, H.: Drug resistance of cancer cells. *Annual Report on Co-operative Research in Cancer (Ministry of Education of Japan)*, 1961, pp 136-141.
- (13) ISAKA, H., SATOH, H., MORIWAKI, A., TASHIRO, T., and SAKURAI, Y.: Studies on the drug resistance of tumors. V. Population analysis of tumors with special respect to the grade of drug resistance. *Proceedings of Japanese Cancer Association, 20th General Meeting, 1961*, pp 22-23.
- (14) YOSHIDA, T.: The nature of acquired drug resistance. *Cancer Chemother Rep* 13: 123-128, 1961.

## PLATES

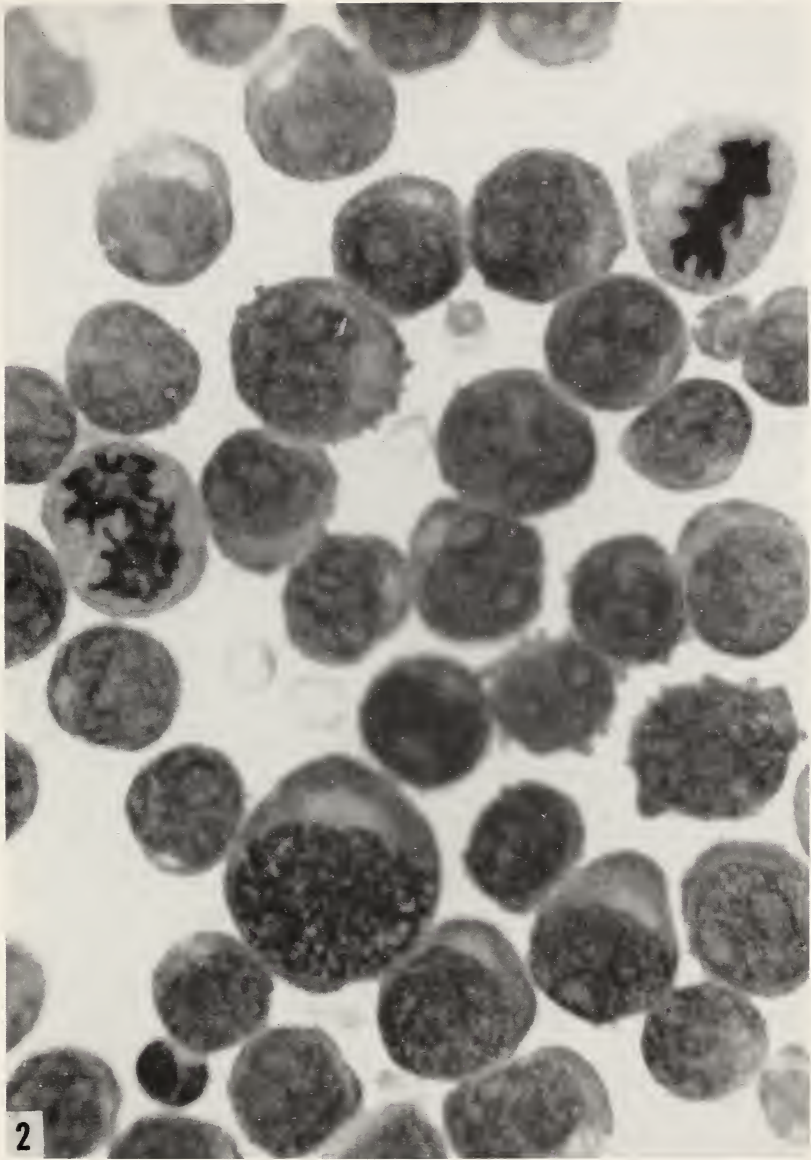
## PLATE 21

FIGURE 1.—Phase-contrast microscopic view of 4-day-old ascites of ascites hepatoma AH 272. Abundant, individually isolated tumor cells with a small number of tumor cell clusters.



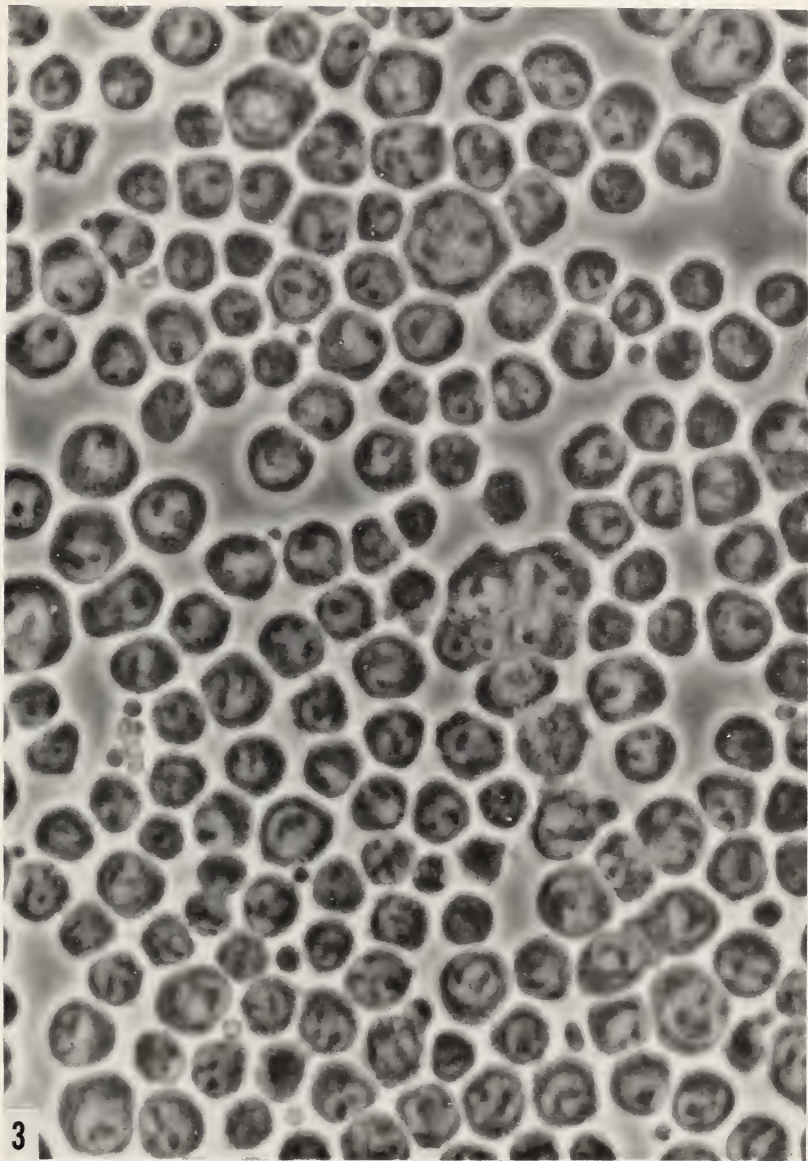
## PLATE 22

FIGURE 2.—High-power view of 4-day-old ascites of ascites hepatoma AH 414, presenting a nearly pure culture of individually isolated cells. Wright-Giemsa stain.



## PLATE 23

FIGURE 3.—Phase-contrast microscopic view of 4-day-old ascites of ascites hepatoma AH 39, showing an abundance of individually isolated tumor cells and a few tumor cell clusters.



3



## **Transplantable Leukemia as an Ascites Tumor: Its Histogenesis and Progress of the Disease**

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**ALTHOUGH** leukemia may be considered a proliferative syndrome of hematopoietic tissues, it is more reasonable that this disease is usually a neoplastic growth. The most convenient tool for the experimental study of such a growth is mouse leukemia which, with some exceptions, is undoubtedly a neoplasm. In such a leukemia, the cells are isolated when they multiply. When neoplastic cells multiply in body fluid in isolation or in a small group of few cells, the condition can be called an ascites or a fluid tumor. In leukemia, cells multiply in blood and also in abdominal or in thoracic fluid. Consequently, it would not be inconsistent to call leukemia a fluid tumor or an ascites tumor in the broad sense of the word.

Transplantable leukemia in mice is clearly neoplastic and the cells, if transplanted into the peritoneal cavity of other animals, would multiply in the ascites and, at the same time, leukemia would develop. Since the disease is an ascites tumor and leukemia, it would be more appropriately called a leukemic ascites tumor. In other words, the other ascites tumor in general differs from the leukemia in only one point—leukemic or not—although the disease pattern of the leukemic growth is not simple and is more variously modified. The persistent nature of the ancestral cell in leukemia may give rise to high leukemic peripheral blood and to preferential invasion into, and destruction of, hematopoietic tissue, which can cause a leukemoid reaction. This reaction is probably one of the major reasons for the complexity of leukemia. Even if such characteristics are present, the principle of leukemia lies in the neoplastic growth of the cells and would not be essentially different from ascites tumors in general.

Development of leukemia must also be essentially the same as the formation mechanism for tissue tumors. In general, the ability of constituent cells in most organs to multiply, however, is usually very low in the normal state; in contrast, hematopoietic tissue is physiologically very active and multiplies rapidly through mitosis. Cells increased by mitosis in time undergo differentiation and maturation and finally die, multiplication and death of cells being kept in balance and resulting in no increase in the number of cells. For example, a verruca, said to be hyperplastic, is caused by a virus. Since basal cells of the skin undergo rapid multiplication and maturation to cornification comparable to those in hema-

topoietic tissue, it would be easier to postulate that some presumably depressing force has been given to the differentiation and maturation phenomena by action of a virus without acceleration of multiplication. If maturation is retarded and cornification (destruction) of cells ceased, then increase in number of basal cells, *i.e.*, hyperplasia, would result. This would not be a neoplasm.

Whatever the cause, retarded maturation of hematopoietic cells is likely to cause hyperplasia, as in skin verruca. In such a case, it is not likely that the phenomenon of hyperplasia would be found only locally, since these cells are wandering and easily movable; the multiplied cells would enter the blood flow, become embedded in other hematopoietic systems, sometimes undergoing growth there, and finally cause a leukemia-like state. If such a phenomenon occurs, it may become difficult to distinguish it morphologically from neoplastic leukemia. These are the characteristics of hematopoietic cells.

Based on such characteristics, various problems connected with development, histogenesis, leukemoid reaction, and tumor-host relation will be described.

## DEVELOPMENT OF MOUSE LEUKEMIA

In general pathology, existence of internal and external causes on etiology is often emphasized and this is no exception in the development of cancer. If the external cause (carcinogen) is strong, the part played by the internal cause would be small. If the carcinogenic action is not so strong, predisposition of the animals would be the determining factor for the incidence. This idea is supported by the fact that, in experiments producing liver cancer by azo-dye feeding, the incidence is high in rats, low in mice, and nonexistent in rabbits. With other external agents, such as X ray or carcinogenic hydrocarbons, a similar idea is reasonable. Some strains of mice contract leukemia when they reach a certain age, without application of any external force. In such instances, predisposition of the animals is almost entirely responsible for the disease, but this does not completely deny the presence of an external action. As long as an organism lives, it cannot avoid the effect of very weak radioactive rays or that of bacteria, and it is not possible to deny these as the cause of leukemia. Consequently, it would be appropriate to think that there is a predisposition so strong as to cause development of leukemia even by such a weak external action. From such a viewpoint, leukemia may be considered to develop by the concerted action of these two factors, but they will be discussed separately.

### External Cause

There are numerous reports on the experimental production of mouse leukemia. Substances known to produce leukemia are carcinogenic

hydrocarbons (1-4), 2-acetylaminophenanthrene (5), and several others (6, 7). Estrogens (8) can produce leukemia but this occurs internally, though given externally. Increase in incidence and acceleration in development of leukemia by the synergetic action of the external causes are also known (9). Leukemia may also be caused by viruses (10-14). The process of leukemic change from external action is, for the most part, unknown and is left for further studies. There is a report on the process of leukemia production in mice by external action of an azo dye (15), examined histogenetically. The results are presented later.

*Initial changes up to 1 month.*—From the beginning of the experiment, changes occurred chiefly in the lymphatic glands and spleen. In the lymphatic glands generally there was moderate swelling, with a high degree of congestion and hyperemia. Histologically, the blood vessels were dilated and filled with erythrocytes. At the same time, many lymphocytes were seen migrating from the follicles to the sinus. After a few days, there was scattered swelling of the sinus endothelium, exfoliation, or necrotic lesion, with regeneration and proliferation in a few reticulo-endothelial cells. With time, immigration of lymphoid cells into the sinus increased, with a decrease in the follicles and medullary cord. These cells migrating into the sinus must be mobilized into the blood (figs. 1 and 2).

Similar changes were seen in the spleen, with degeneration of reticulo-endothelial cells, decrease of lymphocytes, and hyperemia both in follicles and red pulp. Because of these changes, histiocytes, mononuclear cells, lymphocytes, or splenic megakaryocytes probably enter the flowing blood. Corresponding to the cell migration in lymph nodes and spleen, the foregoing cells filled the sinusoid of the liver (figs. 3 and 4), showing histological resemblance to leukemia. Constituent cells infiltrating were mature cells, such as lymphocytes, plasma cells, monocytes, and sometimes megakaryocytes (fig. 4). These megakaryocytes were ordinarily present in the spleen in mice and may have migrated by the described changes in the spleen. Consequently, other cells also may have migrated from spleen and lymph nodes. Similar changes were observed in the adrenals and the lung. Marked increase of white blood cells was seen in the peripheral blood.

These changes in the liver and circulating blood may be termed leukemoid reaction and offer a hint in the elucidation of the mechanism of the reaction.

*Changes up to 150th day.*—Retrogressive changes in the lymph nodes and spleen, such as degeneration and necrosis, still existed and hyperplasia in the reticulum and lymphoid cells in the follicle was observed. There was phagocytosis in the spleen, which was probably a reaction against debris of the cells that had migrated into the flowing blood by the aforementioned changes. Hyperplasia of lymph follicle increased with time (fig. 5). Hyperplasia of lining cells including the Kupffer's cells occurred in the liver and resulted in rounding and formation of a proliferative small lesion of round cells (fig. 6). In some animals, infiltration of

small lymphoid cells into the Glisson's capsule in the liver, peribronchial area in the lung, and the kidneys was observed. These changes were difficult to distinguish from leukemic infiltration and were described as leukemia-like. It was thought that these changes were due mainly to proliferative stimulation of an azo dye, one being a hyperplasia of reticulo-endothelial cells and the other, of lymphoid cells. Such leukemia-like changes were seen in 33 of 43 animals, from a total of 51 examined, excluding 8 with leukemia as a neoplasm.

*Leukemia cases.*—Eight of 51 animals had leukemia characterized as a neoplasm without thymic lymphomas. As would be presumed from the proliferative stimulation given, there were changes in the lymphatic and reticuloendothelial systems (figs. 7 through 10). Both were negative to the oxidase reaction.

It is known that azo dyes generally have the greatest affinity to rat hepatic cells and produce hepatomas. On the other hand, azo dyes are known to cause formation of endothelioma and hyperplasia of Kupffer's cells in mice. Such action may, in some species of animals, produce leukemia before the formation of a hepatoma. Thus, it may be reasonable to think that thymus has almost no part in the formation of leukemia, and this would not result in the development of lymphatic leukemia with thymic lymphomas. The important point is the leukemia-like changes occurring as the precursors of leukemia. Production of leukemia by an azo dye may be schematically represented as follows:

Azo dye→destruction and hyperplasia of reticuloendothelial and lymphatic tissues→mobilization of mature and immature lymphocytes→leukemoid reaction→leukemia-like change→leukemia.

Such leukemia development does not apply to all leukemia but the possibility is strongly suggested. Development of leukemia in this form is characteristic of carcinogenic substances, which differs fundamentally from that caused by X irradiation in which a disposition in the thymus may be the greatest cause of leukemia development.

It should be added that Yoshida sarcoma is assumed to be derived from reticuloendothelial cells (16). The development of leukemia in mice by an azo dye, shown by hyperplasia of the lining cells of liver sinusoid to reticuloendothelial leukemia, is considered as sufficient basis for the origin of Yoshida sarcoma from reticuloendothelial cells.

#### Internal Cause

It is well known that leukemia can be produced in mice by X irradiation (17, 18). X ray is mutagenic, and induction of leukemia in mice can be due to such direct action. If X ray acts as a mutagen and directly causes neoplastic change in hematopoietic cells, there should not be a different incidence in the induction of leukemia by X rays according to mouse strains. There is a high incidence of leukemia in irradiated C57BL and A mice (17), while the rate is very low in mice of other strains. This is inconsistent with the aforementioned direct action of X rays.

Leukemia in C57BL and A mice by X irradiation usually takes the form of thymic lymphoma in which leukemia cells probably originate in thymic lymphoid cells. Development of leukemia by X irradiation is affected greatly by excision of the thymus (19, 20), which indicates the importance of the thymus as a predisposition for leukemia.

There is a high rate of spontaneous development of leukemia in AKR and C58 mice when they reach a definite age. This spontaneous development does not wholly deny an external cause for leukemia. Action of natural radioactive rays, reaction of lymphoid tissue to bacterial infection, and miscellaneous other external causes may have a weak action in inducing leukemia. AKR and C58 mice are greatly predisposed to leukemia and do not require any apparent external cause to develop it. This spontaneous leukemia also often takes the form of thymic lymphomas. Similarly, the high incidence of leukemia can be reduced by excision of the thymus (21). According to Law (22), development of leukemia in DBA mice by a carcinogenic substance is also affected by removal of the thymus.

It may not be entirely satisfactory to take thymus as the sole predisposition for the development of leukemia. Estrogen is well known for its leukemogenic action (8). When a large amount is injected into mice, destruction of mature lymphocytes results, and a marked retardation of maturation process is observed in subsequent regeneration (23). Other known causes include the action of adrenal cortex on lymphatic tissues and effect of the excision of ovary or testes on the rate of leukemia development (19, 24). These facts show that these endocrine substances act as a homeostasis of the lymphatic system, and imbalance of such a homeostatic system would indirectly affect the thymus gland. There is no doubt that a primary predisposition lies in the thymus and this organ may be the most important for the development of mouse leukemia. It is generally assumed that a hormone-like substance, LSF (25), is present in the thymus as the internal predisposition for leukemia, and the stimulating effect of this substance on the proliferation of lymphocytes has already been proved. This substance is said to have a bearing on reticulum cells of thymus cortex which are positive to periodic acid-Schiff (PAS) staining (26).

#### Cytogenesis of Thymic Lymphomas

The next question is how the lymphoid cells develop into leukemia cells. The histologic examination hitherto used was insufficient for satisfactory methodology and results. A new method was therefore devised (27) and was applicable to other fields as well. By this method the composition of thymic lymphatic cells could be followed quantitatively. Only a brief outline of the method will be given.

*Method.*—The thymus was placed in a petri dish. A few drops of physiological saline solution were added and the gland was cut into small pieces with scissors. A few drops of acetic acid-orcein solution were added to the resulting milky fluid containing small pieces of the thymus and

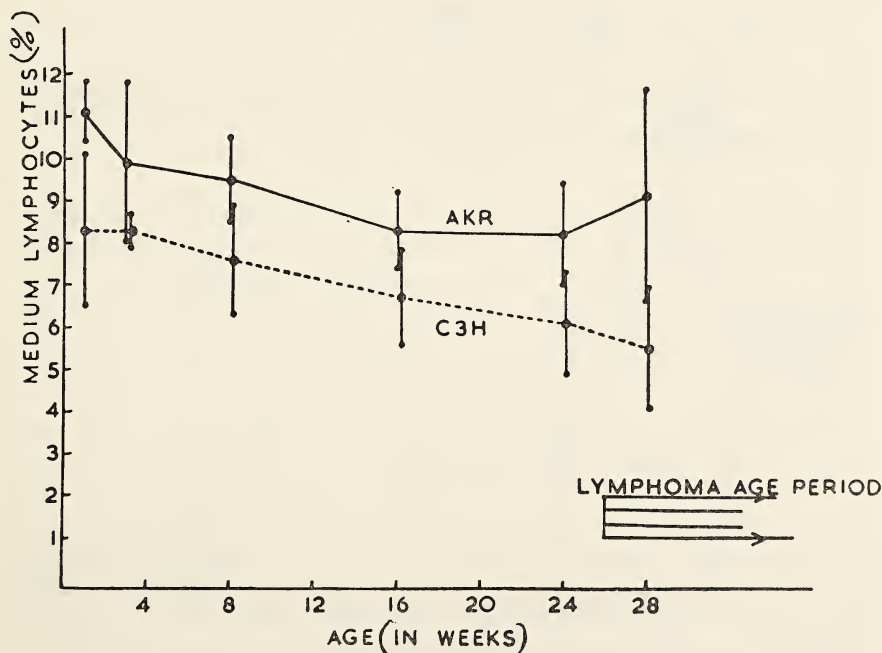
this effected fixation and staining in a few minutes. The small pieces of the thymus were further crushed with forceps before the fixation progressed fully. To obtain a more contrasting specimen, a few drops of gentian violet-acetic acid were added. A drop of this mixture was placed on a glass slide, covered with a coverglass, and mildly pressed with a finger tip to squeeze out excess fluid. Density and stainability of the cells were examined microscopically. If the specimen showed the cells were too dense and staining inadequate for counting the cells, some more staining agent was added. The coverglass was finally sealed with paraffin. Addition of glycerol after staining and storage in an ice chamber preserved the preparation for a few months. The staining agent was prepared by mixing 30 ml of glacial acetic acid with 0.75 g of the dye, and the mixture was heated to dissolve the pigment, cooled, and diluted to 100 ml with distilled water. This was filtered and the filtrate stored until use. Orcein solution produces a precipitate during storage and must be filtered from time to time. Some of the cells may be destroyed, but their maturity can be judged from the structure of their nuclei (figs. 11 through 17).

The materials used for the examination were from AKR mice with a high incidence of leukemia; C3H mice, with a low incidence, were used as controls. Leukemia generally develops in AKR males from the 7th month after birth, and in females from about 6 months after birth. The over-all incidence is 90 to 95 percent. In C3H mice, leukemia incidence is around 3 percent, and death from leukemia generally occurs early, during 4 to 5 months after birth.

A total of 192 mice of these two inbred strains was used for the examination; 8 males and 8 females were killed by ether anesthesia 1 week, 3 weeks, 2, 4, 6, and 7 months after birth. The thymus gland was removed immediately after sacrifice. The same examinations were made with thymus taken 6 and 7 months after development of thymic lymphoma in 10 other animals. Transplantation tests were also carried out on 23 other mice killed at 6 and 7 months after birth. In one thymus, 5,000 lymphoid cells were counted and maturity of the cells was judged by their nuclear chromatin structure (figs. 12 and 17). The cells were classified as large, medium, and small lymphocytes by their degree of maturity, and their number was recorded. At the same time, frequency of mitosis was also recorded and 100 mitotic cells were counted to examine their abnormality. In the classification of these cells, large lymphocytes were considered the most immature, medium lymphocytes were slightly maturing cells that still had mitotic ability, and the small lymphocytes were the completely mature type which would not undergo cell division any longer under ordinary conditions. Although individual differences in judgment cannot be avoided, no claim is made that the absolute values given later are correct. However, the results obtained gave unequivocal data for quantitative variation in the cells according to the age and sex of animals and difference in animal strains.

*Cellular composition.*—There was little variation of large lymphocytes according to age, sex, or strains, but a difference existed between individuals. The rate was around 1.3 to 2.3 percent. With medium lymphocytes there was a definite tendency to fluctuate by age, sex, and strains (text-fig. 1). The highest value of around 11 percent was observed 1 week after birth in both strains. Male AKR mice showed about a 10 percent decrease by 3 weeks after birth and the rate fell to about 8 percent with age. In female AKR mice, the decrease occurred a little later—the lowest rate being 8 percent 4 months after birth. In C3H mice, the decrease by age was much more marked, the lowest rate being around 6 percent in the males and less than 8 percent in the females. These facts show that the ratio of medium lymphocytes is invariably greater in the AKR than in the C3H strain, and larger in females than in males of both strains. The most marked characteristics in the AKR strain was that a great individual difference appeared 6 to 7 months after birth, which was just before the development of thymic lymphomas, and that some individuals tended to accumulate medium lymphocytes. Such a phenomenon was not observed in C3H mice. The significance of this increased number of medium lymphocytes was analyzed by an intravenous transplantation test.

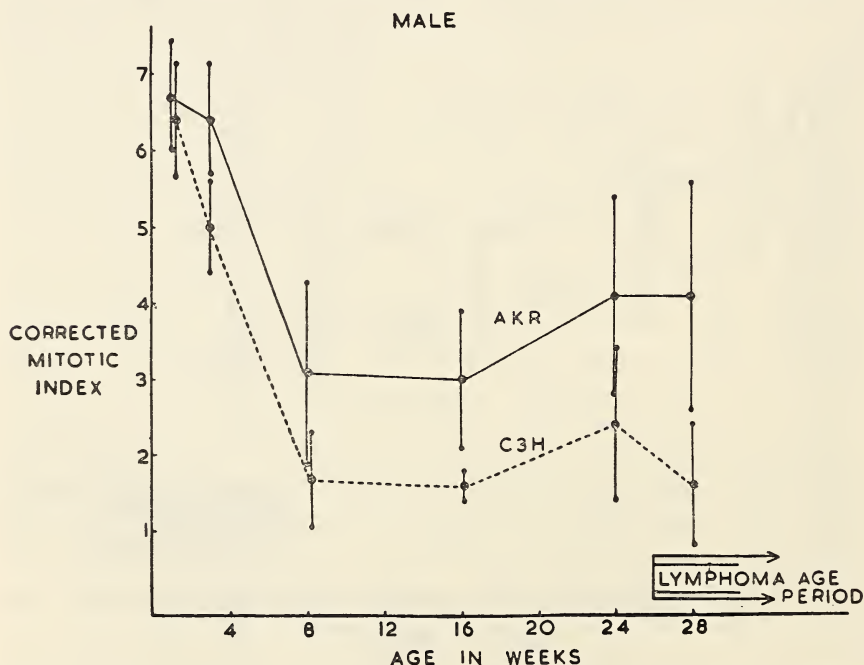
*Mitosis.*—In general, the rate of mitotic cells to total lymphoid cells was higher in the thymus of AKR than C3H mice and greater in females than in males. The rate was  $1.1 \pm 0.1$  percent in 1-week-old AKR males.



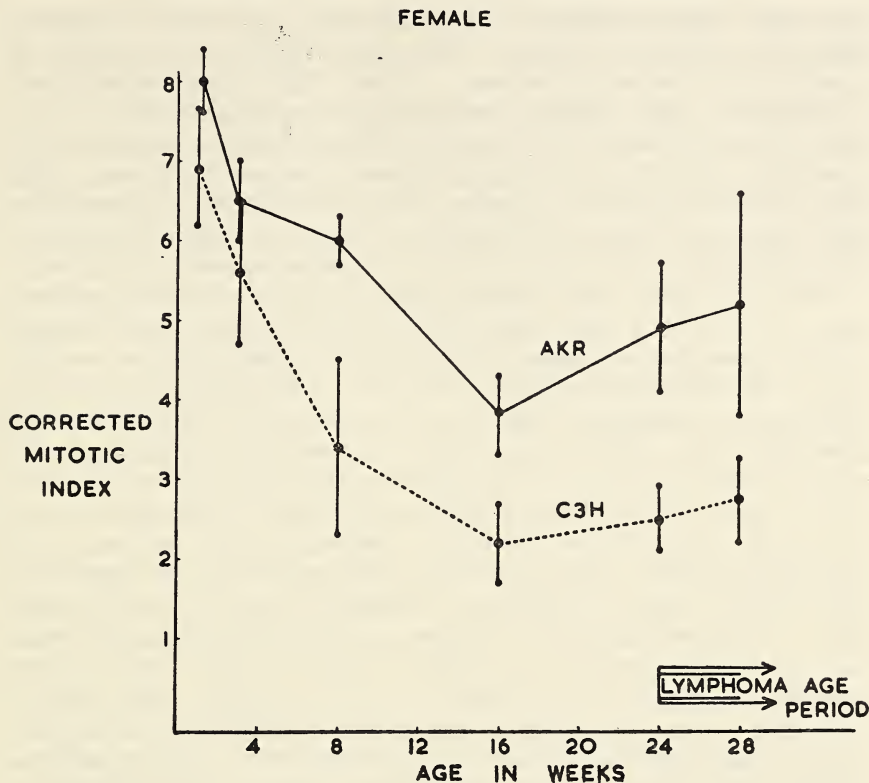
TEXT-FIGURE 1.—Difference in the percentage of medium lymphocytes in thymus of AKR and C3H mice at various ages.

By the 8th week after birth, this rate fell markedly to  $0.6 \pm 0.2$  percent, which corresponded to the period of decrease in thymus weight by age involution. In AKR females, the decrease of thymus weight was small, the period was later, and the decrease of mitotic rate according to age also agreed with this decrease of thymus weight. The rate of mitotic cells increased at 6 to 7 months after birth in both males and females. In the C3H mice with low incidence of leukemia, there was almost no difference between males and females. The rate was high, as in AKR mice, in 1-week-old C3H mice, but there was a sudden decrease from the 3d week and the value became constant at 0.4 to 0.6 percent after 8 weeks, with no increase after 6 to 7 months.

Small lymphocytes are said to have no mitotic ability in a physiological condition (28, 29), and abnormal mitosis that is often observed is generally considered ineffective in increase of cells. These two points are important in a count of the mitotic rate and give the corrected mitotic index (text-figs. 2 and 3). These indexes clearly demonstrate a difference between C3H and AKR strains and between males and females in the AKR strain. The mitotic rate in young animals is as high as 7 to 8 percent, which is almost twice the value of about 3 percent in Yoshida sarcoma cells. It was calculated that lymphoid cells in thymus underwent cell division once in 12 hours in strain AKR and in 20 hours in strain C3H, about the same as in Yoshida sarcoma. Whether the cells



TEXT-FIGURE 2.—Corrected mitotic indexes of primitive lymphoid cells in the thymus of male AKR and C3H mice.



TEXT-FIGURE 3.—Corrected mitotic indexes of primitive lymphoid cells in the thymus of female AKR and C3H mice.

Reprinted from *The British Journal of Cancer* 15: 306, 1961.

increase by repeated division at this high rate depends on whether the divided cells undergo maturation and death or remain immature and alive. Variation in the ratio of cellular composition and rate of mitosis is always higher in the AKR than in C3H mice, and in the females than in males, and the variation agrees with the period of decrease in thymus weight. The important point is that the cell division and proliferation take place at an unexpectedly rapid rate in the AKR thymus, and this might be the chief cause of the neoplastic change in the cells. This phenomenon is specific to the thymus and the value is less than one third to one fourth this rate in other lymphatic tissues.

*Thymic lymphoma.*—The thymic lymphomas that developed may be divided into local and systemic, and both are leukemic. The same examinations were made on 10 mice with thymic lymphoma, and cellular composition was found to be inconstant. Of these 10, 4 had a large number of cells resembling medium lymphocytes, 3 had more than 30 percent leukemic cells resembling large lymphocytes, and 3 had a larger number of small, lymphocyte-type cells. The rate of mitosis was comparatively

low, with a high incidence of abnormal mitosis, the corrected mitotic index being 0.9 to 2.5 percent. This value of about one third that of normal thymus seems rather strange. When 1 AKR mouse was autopsied, a bean-sized localized nodular lymphoma was present. Mitotic rate of this nodule and the remaining uninvolved tissue of thymus were examined separately. Mitotic rate in the lymphoma node was 1.5 percent, while that of the other part was 5 percent. Immature cells were 33 percent in the former and 10 percent in the latter. These values show that the rate of mitosis is far lower in the lymphoma nodes than in normal thymus.

The rate of mitosis does not necessarily agree with the rate of proliferation. In AKR and C3H mice, immature thymic lymphocytes undergo maturation at the same rate as the mitosis and proliferation, *i.e.*, one half of the cells formed by mitosis undergo maturation and death. If the percentage of maturation and death of the daughter cells is in complete agreement, there cannot be hyperplasia. If neoplastic change occurs, the process of maturation and death of cancer cells must be inhibited, at least to a certain extent. If the inhibition is complete, there would be an increase in the number of cells that corresponds to the number of cells undergoing mitosis. Consequently, the cells would increase independently of the rate of mitosis. Inhibition of maturation is not limited to cancer cells. In hyperplasia of tissues in general, first the inhibition of maturation should be considered and then the rate of mitosis. In the latter, insufficient compensatory destruction of the multiplied cells would be necessary. In any case, such an abnormally high rate of mitosis in thymic lymphoid cells could at times be compensated for by the increased rate of maturation and destruction of the cells. However, the possibility of neoplastic change is greater during this period.

*Transplantation test.*—AKR mice not developing leukemia showed vast individual differences in the number of immature cells (text-fig. 1) and rate of mitosis (text-figs. 2 and 3) in the thymus. Possibly the increase of immature cells in these animals were cells that had undergone neoplastic change. To solve this problem the following transplantation experiment was carried out, with the same AKR mice of the same sex, about 1.5 months old. The thymus was removed aseptically from the animals 6 and 7 months after birth, and part was used for the foregoing cytological examination and the other half was injected into the femoral muscle of 4 mice. Twenty-three thymuses were used for this examination. When animals died they were autopsied. Those living were observed for 3 months, after which they were killed and autopsied. Details of the examination are given in (30). Transplantation results were: 1) The so-called positive transplantation, with formation of tumor mass at the site of transplantation and a generalized leukemia, was observed in 6 of 23 animals, in which neoplastic cells existed in the donor thymus. Cytological examination of the donor thymus of 5 of these 6 thymuses showed an increase in immature lymphocytes. There was also a decreased rate of mitosis.

2) In 7 of 23 animals there was no change. Only 1 of these 7 showed an

increased number of immature cells (15.4%) in the thymus used for transplantation. Other values were normal, and the case seemed to be more of an atrophic type.

3) Intermediate between the first two results, there were definite thymic lymphomas, with or without generalization, with no tumor mass at the site of injection and no more enlargement of the draining lymph node than other lymph nodes. This means that pathologic changes equal to those of spontaneously developed thymic lymphomas in AKR mice were found in these animals. There were 10 animals of this type and cytological findings of donor thymuses showed intermediate values of the foregoing two results with no specific changes.

These results indicate that the increased number of immature cells in the thymus of animals 6 to 7 months after birth is due mainly to neoplastic cells. The third type was unexpected and hard to explain, but offers a useful basis for elucidation of the mechanism of the neoplastic changes in cells. The most probable explanation would be spontaneous development of thymic lymphomas, but because of the 39 animals receiving transplants only 15 had developed thymic lymphomas when they were autopsied at 18 weeks of age, this is unlikely. In the control AKR mice, only 1 of about 100 autopsied at the age of 22 weeks had a medium-sized thymic lymphoma. Since there had been 15 of 39 animals 1 month younger with lymphoma, the possibility of spontaneous development can be ruled out.

The second possibility is a viral agent, which is less likely from past virological reports on AKR mice. The third possibility is the assumed presence of cells intermediate between normal cell and neoplasm. Berenblum (31) assumed a "dormant malignant cell" in his experiments on the development of skin cancer.

It may be assumed that a certain amount of maturation inhibition is present in the cells of such a hyperplastic locus. If the neoplastic change were closely related to the inhibition of maturation, the cells may be those having lost the ability for differentiation, may be partially changed, in the intermediate stage of the change, or ready for neoplastic change.

Whatever the expression used, it may be assumed that such an intermediate type of cells between the normal and neoplastic had migrated from the site of injection through the body and had reached the thymus. As stated above, there is an accelerative action in the thymus for mitosis of lymphocytes (22, 25). In the AKR mice, such action continuing for over 6 months after birth is assumed to result in the change of thymic lymphoid cells. If such an action on the intermediate-type cells reached the thymus, the change must take place earlier than that of normal thymic lymphoid cells. Such intermediate cells that have migrated and settled in other parts of the body, such as the injection site or lymphatic glands draining the site, would be free from the action and would not undergo the change. Consequently, a primary thymic lymphoma would develop in a few months. There may be other possibilities, but such a hypothesis is attractive.

### Viral Leukemia

In recent years, virus has been increasingly noted as the cause of mouse leukemia. Virus in a narrow sense, such as that of smallpox or poliomyelitis, would lead to misunderstanding. Whatever the definition, it is impossible to deny the development of leukemia or leukemia-like neoplastic disease by a virus or virus-like particles. Polyoma (13), Moloney (12), and Friend (10) viruses are well known. Mechanism of the development of leukemia by these viruses is still being investigated. The neoplastic nature of Friend's disease caused by Friend virus is still doubtful and the term "leukemia" is never used for it. The disease can be reproduced in a new animal by injection of homogenized spleen of a diseased animal. Local proliferation of the cells at the injected site is negligible and the virus contained in the homogenate is assumed to cause hyperplasia of hematopoietic tissue, especially of splenic cells (10). What would be the action mechanism of the virus in such a case; would it be the acceleration of cell division? As was stated in the section on the physiology of the thymus, even a high rate of cell division, such as once in 12 hours, is compensated for by the acceleration of cell destruction by the homeostatic regulation to inhibit hyperplasia of the tissues for a few months. If the action of the virus is merely acceleration of cell division, it would be natural to think that compensation and proliferative disease like Friend's disease should not develop within 1 month or so. It would also be impossible to think that a virus could accelerate more than at such a high rate. The most rational explanation is that the primary action of a virus is the inhibition of maturation and that acceleration of cell division, if any, is a secondary action of the virus.

If maturation inhibition causes hyperplasia of hematopoietic tissue, the multiplied cells would enter into the blood stream and be distributed all over the body because of the limited volume of bone marrow or organs and isolation of the cells, and this might finally cause leukemia-like pathological change. Undoubtedly, this hypothesis offers a good subject for future research.

### LEUKEMIC ASCITES TUMORS

In general, leukemia is said to be neoplastic proliferation of hematopoietic cells and there is also a non-neoplastic disease that cannot be distinguished morphologically from neoplastic leukemia. If these two are to be called leukemia, its characteristics would be the proliferation of hematopoietic cells and their distribution through the blood stream, with formation of tissue infiltration. For discussion one material either tumorous or nontumorous would be sufficient. In practice, the most convenient is a transplantable neoplastic leukemia.

#### Leukemia-Like Nature of Rat Ascites Tumor

*Yoshida sarcoma*.—Yoshida sarcoma originates in Kupffer's stellate cells which resemble monocytes (16, 32). When transplanted into the

peritoneum, the cells proliferate in the peritoneal fluid and the form is known as ascites sarcoma. When appropriate animals for its growth are used, a few tumor cells appear in the peripheral blood during the last stage of the disease. Since there is little leukemic infiltration, the disease cannot usually be termed leukemia. Experiments on intra-peritoneal transplantation of Yoshida sarcoma into various pure strains of rats have shown that there is a wide difference in the transplantation rates, with few leukemic characteristics (33). In a few transplanted Yoshida sarcomas into Donryu rats, marked leukemic characteristics were observed, such as the appearance of tumor cells in the peripheral blood and formation of leukemic infiltration. According to Honjo *et al.* (34), transplantation of Yoshida sarcoma into the bone marrow of Gifu and Saitama rats resulted in leukemic infiltration. Appearance of tumor cells into the peripheral blood differs in the two strains and it was concluded that Yoshida sarcoma is a monocytic leukemia and that inherent resistance of animals controlled the progress of leukemic characteristics.

*Hirosaki sarcoma.*—According to studies by Usubuchi *et al.* (35, 36), the cellular form of Hirosaki sarcoma is similar to that of Yoshida sarcoma. Its biological characteristics, such as the formation of tumor mass in the peritoneum, are also similar to Yoshida sarcoma, but Hirosaki sarcoma is more characteristic of leukemia than Yoshida sarcoma, such as the appearance of tumor cells. (Usually the leukocyte count was over 60,000 and tumor cell number was over 70%.) Usubuchi *et al.* obtained a subline strain with stronger leukemic characteristics.

*Transplantable leukemia.*—Nakamura reported a transplantable strain of leukemia which had developed in rats treated with an azo dye and Nitromin (37, 38). The cells were similar to the mature lymphoid type. When transplanted, the only tumors produced were soft, pyoid, and easily crushed to form a milky fluid. The cells appeared abundantly in peripheral blood and the number of tumor cells reached over 90 percent in most cases. This strain had a very low rate of transplantation and finally became extinct. This strain seemed to have stronger leukemia characteristics than the foregoing two.

The difference in leukemic characteristics among the three strains is due to the intrinsic nature of their cells, but it must be remembered that this difference may appear as leukemia or tumor according to the difference in the host's condition. In short, the disease type appearing in an individual is limited by two conditions: the nature of the cells and condition of the host.

### Mouse Leukemia SN-36

There are many transplantable mouse leukemias. It might be necessary to describe each of these, but a general description of one strain will give their common nature and their handling. Mouse leukemia SN-36 will therefore be taken as representative of all transplantable leukemias, and its nature, formation of tissue infiltration, and disease type will be discussed.

*Development*

Leukemia may develop by treatment of the animals with nitrogen mustard or its derivatives (7), but no transplantable strains have been obtained. Nakamura (39) obtained a transplantable strain by the following experiment: Commercial dd mice, 180, were given 11 intraperitoneal injections of 0.25 mg of Nitromin with an interval of about 1 week. Twenty-three mice lived over 6 months and were histologically examined. Deaths of mice before 6 months were due mostly to the toxicity of Nitromin and in these animals, 22 of 23 showed leukemia-like changes. One showed a marked accumulation of ascites and an intensive anemia 1 year after the start of the experiment. The lymphocyte-like, small, round cells in the ascitic fluid were in the state of almost pure culture. A transplantable strain was obtained from this animal.

*Observations of the primary animal*

Leukemic swelling was observed in the liver, spleen, and lymph nodes, together with an accumulation of ascites and severe anemia. There was no formation of thymic lymphoma. Erythrocyte count was  $6.5 \times 10^6$  cells per  $\text{mm}^3$ , indicating an intensive anemia (erythrocyte count of normal mice is around  $10^7$  cells/ $\text{mm}^3$ ), leukocyte count was 916,000, and leukemia cells (hereinafter designated as tumor cells) were 98 percent. Oxidase reaction of the tumor cells was negative. Leukemia-like tissue infiltration in the lymph nodes was most marked in cortical follicles, replacing the medullary cords, and was comparatively slight in the medullary sinus. Infiltration was found also in the cortical sinus and capsule, spreading to the surrounding soft-tissues and forming a tumor. There was an enlargement of lymph follicle and medullary cord in the spleen, and these were filled with the tumor cells. Infiltration was comparatively slight in the red pulp, where splenic megakaryocytes remained. Such infiltration of the lymph nodes and spleen may be called typical of infiltration of lymphoid leukemia. There was an infiltration in the liver surrounding the Glisson's capsule, and diffuse infiltration of approximately the same degree was found in the acinus. Infiltration in the lung was mostly in the peribronchial interstitial tissue and was also found in the alveolar septum. Diffuse infiltration was observed in the kidneys.

*Transplantation*

The ascites of the primary animal and ground mush of its lymph nodes and spleen were injected in the peritoneum of 7 dd mice. These were serially transplanted to the 360th generation. The ascites from the first-generation animal 4 days after the transplantation showed an almost pure culture of cells. In 5 mice, tumor cells decreased after 7 days and increased in 2. The cells in the ascitic fluid were transplanted into the peritoneum of 5 dd mice. In 7 first-generation animals the tumor cells disappeared, but recurrence was observed in 1 after 87 days and this animal died with the same autopsy findings as those of the primary

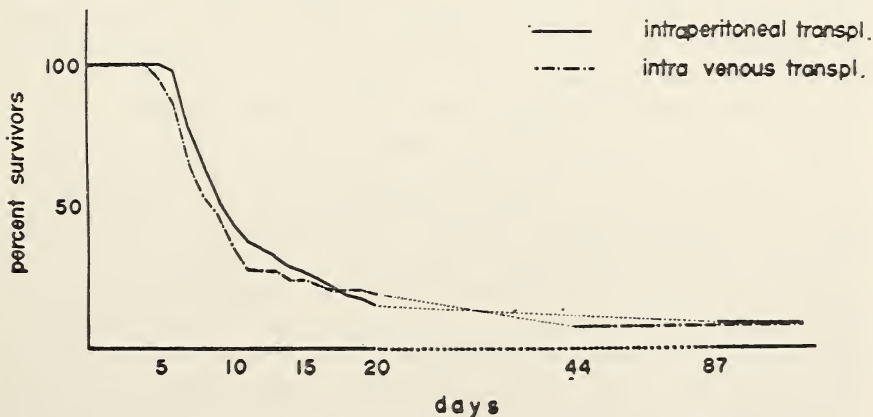
animal. Of the 5 second-generation transplanted animals, 3 underwent spontaneous regression and 2 showed accumulation of tumor ascites, with formation of a small tumor in the intra-abdominal soft tissue and in the abdominal wall. There were leukemic infiltrations in parenchymatous organs and increase of tumor cells in the peripheral blood, and the animal died with a leukemia-like ascites tumor.

Text-figure 4 gives the transplantation rate and survival days of 1,143 animals with intraperitoneal transplantation up to the 205th generation and of 100 animals with intravenous transplantation. Tumor death occurred from the 6th day after transplantation into the peritoneal cavity, 50 percent mortality occurred after 8 to 9 days, and transplantation rate was around 92 percent (results with commercially available dd mice). Intraperitoneal transplantation with 1 tumor cell was positive in only 1 of 6 cases. Transplantation rate was lower with subcutaneous transplantation, but the animals succumbed to leukemia with increase in the size of tumor at the transplanted site. There was no change in the dd mice fed with a piece of living tumor.

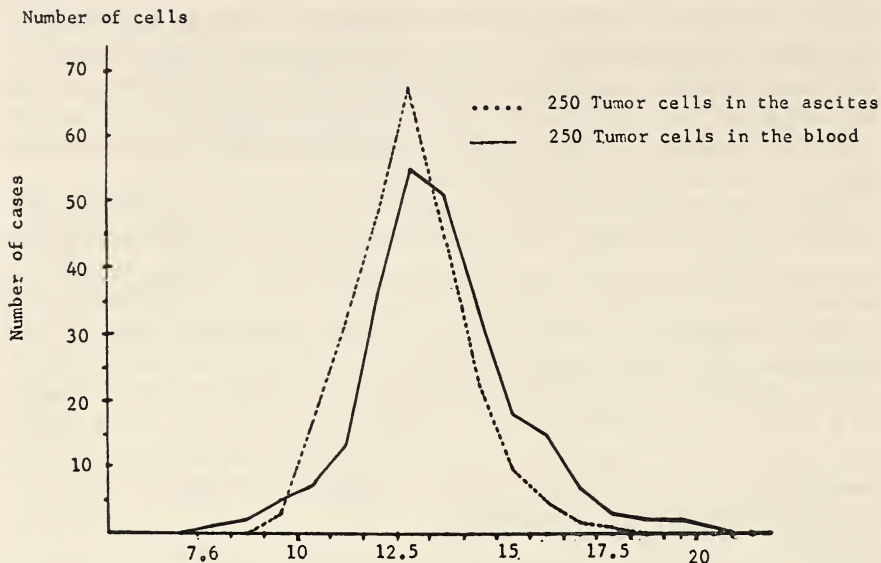
### Cells

Figures 18 through 22 show the form of the tumor cells. Giemsa-stained smear specimen of tumor cells in the ascitic fluid and peripheral blood appeared round or oval, and irregularly shaped in rare cases. The amount of cytoplasm was comparatively small and tended to stain dark, with no stainable granules.

The nucleus showed a slight lobulation or sharp indentation, and chromatin net was slightly rough. There were one or few nucleoli. Observation of living cells under the phase contrast microscope showed the characteristic forms, with abundance of rod-shaped or particle-like granules in the cytoplasm, which were proved to be mitochondria. There was no mobility of the cells and no phagocytic ability. Both oxidase and peroxidase reactions were negative. The size of the cells in the ascitic



TEXT-FIGURE 4.—Percentage survival of dd mice with leukemic ascites tumor SN-36.



TEXT-FIGURE 5.—Fluctuation of the diameter of tumor cells in ascitic fluid and peripheral blood.

fluid was 9 to 19 $\mu$  in the Giemsa-stained preparation and those in the peripheral blood, 7.6 to 20 $\mu$  (text-fig. 5). Their mode was at 13 $\mu$  in both. About 3 percent of the cells in the ascitic fluid showed mitosis and there was hardly any degeneration, though a considerable number of degenerated cells was found in the peripheral blood, with loss of stainability and anuclear cells in some cases. Mitotic figures in the blood were seen in about 1 percent, which was about one third of those in the ascitic fluid.

#### *Increase of the cells*

*Intraperitoneal transplantation.*—Cells transplanted into the peritoneum begin to multiply floating in the ascites. The results obtained with commercially available dd mice will be described later. Pure cultures are attained 3 to 4 days after transplantation when infiltration into the lymph nodes and interstitial tissues of the liver occurs. Later, the cells appear in the peripheral blood and the blood becomes leukemic.

Growth rate of the cells after transplantation of  $10^7$  cells is shown in table 1. Usually the number of Yoshida sarcoma cells at this stage is 2 to  $3 \times 10^5$  cells per mm<sup>3</sup>, but cells of SN-36 are smaller and their concentration in the ascites is greater, reaching  $10^6$  cells per mm<sup>3</sup>. If growth in the ascites alone is considered, this tumor may be considered as an ascites tumor, entirely the same as Yoshida sarcoma. Even in intravenous and subcutaneous transplantation, the cells appear in the ascitic fluid when tissue infiltration occurs and the ascites is in a state of pure culture, but the volume and density are low. In a few exceptional cases, tumor cells in the ascitic fluid disappear, tumor infiltration into the intra-abdominal soft tissue and tumor formation occur, and the animal dies.

*Invasion of tumor cells into flowing blood.*—Intraperitoneal transplantation of the cells results in growth of the cells in the ascitic fluid and appearance of tumor cells in the peripheral blood (table 1). Blood to be drawn for this examination was usually obtained by injection of a glass capillary into the retrobulbar venous plexus. The earliest appearance of tumor cells into the peripheral blood was just 2 days after the transplantation and about 1 percent of tumor cells was found, the number gradually increasing later. The largest number of tumor cells was found on the 6th day, when the leukocyte count was 80,000 per mm<sup>3</sup>. Some animals had over 40 percent of tumor cells and such animals were usually found dead the following day. There were some among the large number observed that showed no leukemic changes, but they are not included in table 1. In the initial period, indicated as 0 percent of tumor cells in the table, a few tumor cells were usually found if the smear specimen was examined in detail. Even if tumor cells were not found in the specimen, intraperitoneal transplantation of 0.01 ml of blood immediately after being drawn gave a positive result. In a few cases, the cells multiplied only in the abdominal cavity, without any leukemic change and without any leukemic involvement of the organs.

*Intravenous transplantation.*—When 1 to 10<sup>6</sup> cells were injected into venous blood, the cells passed through the alveolar capillary of the lung rapidly and multiplied in the liver and lymph nodes. Smear specimens of peripheral blood immediately after intravenous transplantation were less likely to show tumor cells. A count of the cells in the peripheral blood showed that they disappeared after intravenous injection but reappeared after 2 days. The concentration of the cells reached an average of 3.2 percent after 4 days in intravenous transplantation (1.1% in intraperitoneal transplantation). After 5 days, the average concentration in the former was 12.1 percent and 2.7 percent in the latter (tables 1 and 2). The blood then became highly leukemic, there was tissue infiltration, and the animal usually died. But occasionally leukemia did not develop or the tumor cells that appeared transitorily in peripheral blood disappeared completely; a small nodule was formed under the skin, followed by tumor growth. Some of these animals developed leukemia (leukosarcomatosis or tumor-forming leukemia type), and the remaining died without becoming leukemic; this type is called nodular tumor-forming. It is usual that animals with this type live longer (fig. 45).

Intraperitoneal transplantation of a piece of the tumor of such exceptional types, described in the present and previous sections, results in growth of leukemic ascites tumor. It may therefore be said that there has been no irreversible change in the nature of tumor cells themselves.

Correlation between the variety of growth type and survival curve (text-fig. 4) shows that almost all the animals were leukemic if they died within 10 days after the transplantation. If they survived longer, there would be ascites tumor or solid-tumor formation without leukemic involvement.

TABLE 1.—Increase in tumor cells in ascitic fluid and peripheral blood of mice transplanted with SN-36 intraperitoneally

Days after trans-plantation	Num-ber of cases	Ascitic fluid				Peripheral blood			
		Number of cells/mm <sup>3</sup>			Tumor cells (%)	Number of white cells			Tumor cells (%)
		max	min	mean		max	min	mean	
1	10	1.3 × 10 <sup>5</sup>	6 × 10 <sup>4</sup>	8 × 10 <sup>4</sup>	13.0	8.8	9.8	—	0.0
2	10	3 × 10 <sup>5</sup>	8 × 10 <sup>4</sup>	1.7 × 10 <sup>5</sup>	79.3	54.4	69.2	—	0.2
3	10	4 × 10 <sup>5</sup>	2.3 × 10 <sup>5</sup>	3 × 10 <sup>5</sup>	92.3	83.2	87.3	1.2 × 10 <sup>4</sup>	0.4
4	10	6 × 10 <sup>5</sup>	3 × 10 <sup>5</sup>	5 × 10 <sup>5</sup>	97.3	92.5	95.0	1.5 × 10 <sup>4</sup>	1.1
5	10	10 <sup>6</sup>	6 × 10 <sup>5</sup>	8 × 10 <sup>5</sup>	96.4	95.3	97.0	3.9 × 10 <sup>4</sup>	2.7
6	10	1.2 × 10 <sup>6</sup>	8 × 10 <sup>5</sup>	9 × 10 <sup>5</sup>	99.3	97.7	98.8	4.5 × 10 <sup>4</sup>	15.5
7	10	1.4 × 10 <sup>6</sup>	6 × 10 <sup>5</sup>	9.5 × 10 <sup>5</sup>	99.8	98.0	99.1	7.4 × 10 <sup>4</sup>	31.5
8	10	1.3 × 10 <sup>6</sup>	9 × 10 <sup>5</sup>	10 <sup>6</sup>	99.8	99.2	99.5	1.2 × 10 <sup>5</sup>	38.4

TABLE 2.—Increase of tumor cells in peripheral blood (intravenous transplantation)

Days after transplan- tation	Number of cases	Tumor cells (%)		
		max	min	mean
4	20	9.4	0.0	3.2
5	20	49.6	0.2	12.9
6	18	50.4	0.2	25.2
7	14	79.0	1.0	37.8
8	11	60.0	4.0	41.8

Subcutaneous transplantation of the cells resulted in the formation of a tumor at the transplantation site, and appearance of tumor cells in the peripheral blood was usually delayed longer than in the foregoing two methods of transplantation. In some cases, the cells never appeared in the blood and survival was generally longer.

#### *Formation of tissue infiltration*

Whether the injected cells were transported by the blood, fixed in a tissue, and multiplied to form an infiltration focus or whether a new kind of cell started from the host's cells was an important question in connection with the idea of the viral origin of this tumor. The most convenient and reliable method for resolution of this question seemed to be the injection of tumor cells through the tail vein and histological examination of the organs after periodical killing of the animals. This method was therefore followed with injection of about  $10^6$  cells per animal.

*Lung*.—About 98 percent of tumor cells injected into the tail vein was detained as an embolus in the lung capillary (fig. 23). The period of the detention, however, differed markedly according to the nature of the cells (40). For example, cells which formed an aggregate like ascites hepatoma never passed through the capillary at once and most of these cells later underwent degeneration at the embolic site (41). Only a few underwent remultiplication at the site and formed a proliferating lesion—metastasis. The free-cell tumor, ascites hepatoma AH-13, also did not pass through the lung (41) and showed a similar behavior. On the other hand, SN-36 hardly ever showed cell degeneration and most cells passed through the capillary from 0.5 to 1 hour after the injection; their presence was difficult to prove histologically 3 hours later (fig. 24). Such facile passage of the capillaries was due mostly to the small size of the cells but also to the specific nature of the cells originating from hematopoietic cells.

Formation of filtrative lesion in the lung was hardly seen for a few days after the injection, but an infiltration focus was formed late in the disease. This was because the cells which had gone into other organs, particularly the liver, spleen, lymph nodes, etc., and multiplied there again entered the blood stream and were transported to the lung. At autopsy of the animal with tumor, there were two different types of lung

infiltration. One was the formation of a beadlike, diffuse infiltration in the interstitial tissue in the alveolar septum or as an embolus in the alveolar capillary (fig. 25). At the same time, there were scattered small nodules in the parenchyma. In some cases, histological observation of the diffuse-type infiltration was far more intense than that immediately after the injection of  $10^8$  cells through the tail vein, after which the animal usually died of asphyxia.

The other type was a highly localized infiltration in the peribronchial and perivascular interstitial tissue, with only a slight infiltration in the alveolar septum (fig. 26). There was also a combination of these two types. The first was found mostly in the animals with high leukemic blood while the mixed type appeared in the animals with a smaller number of tumor cells in the peripheral blood.

*Liver.*—The cells passed through the lung and were distributed throughout the body after injection into the tail vein. A few cells were found in the sinusoid and Glisson's capsule of liver slice of an animal killed 10 minutes after injection of the cells (figs. 27 and 28). This amply illustrates the rapid passage of these cells through the lung, but it is not certain whether this is a characteristic of leukemia cells. Three hours after the injection, the tissue specimens revealed the reaction due to these cells and formed a small lesion composed of small, round cells, mononuclear cells and eosinophils, surrounded by neutrophils and lymphocytes, scattered throughout the interstitial region and parenchyma (fig. 29). This reaction had disappeared by 12 hours after the injection and tumor cells had disappeared from the sinusoid in the liver after 24 hours. This might have been due to their outflow. On the contrary, considerable accumulation of tumor cells with abundant mitosis was found in the Glisson's capsule and there were almost no reactive cells. Leukemic infiltration focus continued to enlarge after the 48th hour (fig. 30). Such formation of leukemic infiltration demonstrated that the injected cells themselves underwent proliferation. After 4 days, localized infiltration of a medium degree was formed in the Glisson's capsule, but a few cells were still found in the sinusoid. This period coincided with the time of reappearance of tumor cells in the blood.

Autopsy findings in the livers of animals with tumors can be divided into two types: One is the so-called typical figure of the infiltration of lymphoid leukemia which shows circumscribed infiltration in the Glisson's capsule, and the other is a diffuse infiltration in the parenchyma of the liver, which coincides with the so-called typical liver infiltration of myeloid leukemia (figs. 31 and 32). In this experiment, the two types seemed to appear according to the difference of the host animal, since the tumor cells injected were identical. The fact that such two different types were formed from the same tumor cells is of importance and this point will be taken up in a later section.

*Lymph nodes.*—Since there are immature lymphoid cells already in the lymph nodes, which are difficult to distinguish from the cells injected, it is difficult to identify the small number of cells that migrate there in the

initial period. In the animal autopsied 24 hours after the injection, small aggregates of what appeared to be tumor cells were found in the germinal center of the cortical follicle.

After 48 hours, distinct evidence of infiltration of tumor cells into the germinal center was recognized (fig. 33). After 72 to 92 hours, the degree of leukemic infiltration was somewhat increased, and the cortical follicles were replaced by the infiltrating tumor cells. This infiltration seemed to progress gradually into the medullary cords. Immigration of normal lymphocytes into the sinuses was also found (fig. 34). The degree of immigration corresponded to the degree of infiltration. When almost all parts of the lymph follicles and medullary cords were replaced by leukemic cells, a number of normal lymphocytes and tumor cells were found in the sinuses (fig. 35). At this stage, also, lymphocytosis in some cases, and evidence of tumor cells in others, was found in peripheral blood. Subsequently, the number and size of infiltrating foci increased gradually until, finally, recognition of the original structure of the lymph node became impossible. In its final stage, the lymph node corresponded in size to the Azuki bean.

In the case of subcutaneous transplantations, the manner of infiltration into the regional lymph nodes was quite different from that mentioned. About  $10^6$  tumor cells were inoculated subcutaneously into the foreleg of several test animals. After 24 hours, foci of a small number of tumor cells were found in the cortical sinuses of all the animals (fig. 36). Subsequently, there was progressive migration between the cortical follicles into the medullary sinuses. In its final stages, the result of subcutaneous transplantation was difficult to distinguish from the consequence of intravenous transplantation (fig. 37). The final size of the regional lymph nodes was greater than that of soybeans.

In summary: 1) Four days after intravenous inoculation, tumor cells were detected again in the circulating blood. At that time, tumor cells were also detected for the first time in the liver sinusoids and in the medullary sinuses of the lymph nodes. 2) Various types of infiltration were recognized. 3) The progression of lesions in the lymph node depended upon the site of the first immigration of tumor cells.

*Spleen, bone marrow, etc.*—It was difficult to determine in which part of the spleen the injected cells first stayed to start multiplication. Initial infiltration was found in the follicles in most animals, but subsequent growth was not necessarily constant. Some cells multiplied around the follicles, and others underwent infiltrative proliferation in the red pulp (figs. 38 and 39). There was a lack of any correlation with infiltrative form in other organs.

Infiltration into the bone marrow was comparatively rare. In many animals, a marked enlargement of the sinus was observed, with scattered lesions of hemorrhage, tumor cell emboli in arterial capillaries, and degeneration of endothelial cells of the sinus. In such cases, immature myeloid cells appeared in the peripheral blood. Histological examination at death revealed the presence of normal hematopoietic tissues in most

cases examined. In a few, hematopoietic cells of the granulocytic and erythrocytic systems remained in the tissue in small numbers, and a large number of immature myeloid cells and tumor cells were seen in the sinus (fig. 40).

Usually infiltration is not observed in the brain except for a slight infiltration into the pia mater. However, it seems certain from the following experiment that the injected cells migrated into the brain and stayed there. Artificial perfusion of the brain with a solution containing SN-36 cells and other tumor cells through the aorta resulted in the formation of a large number of tumor cell emboli in the capillaries, though injection of SN-36 cells from the tail vein failed to reveal the formation of an infiltrative lesion in the brain. This fact was considered to show the rapid outflow of the injected cells and will be the subject of future studies.

The injected cells invaded the myocardium and took an elongated form, according to the direction of muscular fibers of the myocardium. It is still uncertain whether the cells underwent deformation by the fixation procedure.

In the kidneys, tumor cell emboli were found in the glomerular capillary immediately after the injection. Subsequently, the cells seemed to infiltrate the areas surrounding the glomerulus and tubules, and finally formed a diffuse infiltration (figs. 41 and 42). Intensive infiltration of tumor in the pancreatic tissues was also found (fig. 43).

SN-36 cells injected passed rapidly through the lung, were fixed in various tissues, multiplied there, and entered the blood stream. It should be emphasized that the injected cells underwent proliferation and that the new growth is not the cells of the host animals.

#### *Diversity of disease pattern of SN-36*

The cells of SN-36 injected into mice proliferated in over 90 percent of the dd mice and finally killed the host animal. The tumor growth in the animal until its death, *i.e.*, from leukemia, varied greatly, both macroscopically and histologically, according to the individual differences of the animals (2,000) used.

The progressive form of the disease observed in SN-36 tumor has been classified in 5 types, listed in table 3 (42).

Type I (table 3) is the leukemia that occurred on injection of the tumor

TABLE 3.—Growth characteristics of the leukemic ascites tumor SN-36 in dd mice

Growth type	Site of tumor growth			
	In blood	In ascites	Leukemic invasion in organs	Formation of solid tumor
I. Leukemia	+	—	+	—
II. Leukemic ascites tumor	+	+	+	—
III. Leukemia with solid tumor	+	—	+	+
IV. Ascites tumor	—	+	—	+ or —
V. Solid tumor	—	—	—	+

cells into the blood. This type was characterized by the formation of a leukemic infiltration in the organs and appearance of the cells in the blood, without accumulation of ascites or nodular growth (fig. 44). This might be called leukemia in the narrowest sense of the word.

Type II is the leukemic ascites tumor and occurs most frequently by cell transplantation into the peritoneum. Proliferation occurs chiefly in the ascites with dispersion throughout the whole body. There is little nodular growth under the skin of the belly, punctured for inoculation (fig. 44). There is usually an infiltration into the soft tissue of the peritoneum but little formation of a large tumor as in Yoshida sarcoma. Tumor cells in the ascitic fluid occur also by intravenous transplantation; toward the final stage of the disease there is a pure culture of tumor cells. In such a case, the volume of the ascites and concentration of the cells in the ascitic fluid are very small and the type need not be called ascites.

Type III is the leukemic solid tumor and occurs most frequently in subcutaneous transplantation, though exceptions are found after intravenous transplantation.

These three types are all leukemic and are accompanied by leukemic involvement of the organs.

Types IV and V are ascites or solid tumors, characterized by localized growth of the tumor at the inoculated site, but there is no leukemic generalization. In types IV and V, survival period of the animals is usually 10 days or more and the types are found in 20 percent of the total cases. In the exception, an aleukemia type with formation of a subcutaneous tumor is found after intravenous injection (fig. 45).

These 5 types can be divided further by histological findings. In considering the different forms of the organ infiltration, type I can be divided into two subtypes, one having a tendency for localization of infiltration into organs, and the other forming a lesion of diffuse infiltration. Types II and III can also be divided into two subtypes each, similar to type I. If other observations are added, such as the number of tumor cells in the peripheral blood, an increase of reactive leukocytes will produce limitless variety of classifications. Such a variety of disease is produced by injection of SN-36 cells, which have a constant nature, into a large number of animals. Why is there such variation in the progress of the disease, despite the fact that the cells undergoing multiplication have a fixed and constant character? One reason may be the difference in the site of injection. Subcutaneous injection produces mostly solid tumors or leukemic solid tumors, while intraperitoneal transplantation produces ascites or leukemic ascites. Injection into the blood stream sometimes produces solid tumors, with tumor growth without leukemic change in the organs, and this cannot be explained merely by the difference in the site of injection. If the nature of the cells is identical and difference in the site of injection is not considered, then the other factors causing such a variety of the disease type must lie in the host animal (43) (see the paper on the transplantability of Yoshida sarcoma by Hiroshi Satoh, this Monograph).

*Conditions of the host that control the disease type*

When the tumor cells enter the host body and begin multiplication, the cells must be receiving some kind of action from the host, usually that of nutrition. There may also be an inhibition of growth by immunity. The growth of tumor cells must be highly affected by the suitability or unsuitability of the host. The most unsuitable condition would be the implantation of tumor cells into a heterologous individual, in which case the cells would finally die out. Such condition of the host is generally expressed as the strength of "resistance" of an individual against the tumor growth. Effect of the degree of such resistance on the leukemic proliferation of SN-36 was examined.

(a) In the heterologous body of a Donryu rat, conditions for the proliferation of injected SN-36 cells are markedly poor and the cells will generally die out as other tumors do, but if the number of cells injected into the peritoneum is over  $10^7$ , the cells undergo proliferation and 25 percent of the rats die of tumor. In such a case, serial transplantation into another rat shows the same effect. Retransplantation of the cells from such rats into mice induces the growth of the tumor SN-36, which indicates that there has been no change in the nature of the tumor cells. Histological examination of the rats dying of tumor SN-36 also shows destructive invasion of tissues as a malignant tumor. In any case, the cells tend to form a solid tumor and there is no leukemic proliferation.

(b) Intraperitoneal transplantation of about  $5 \times 10^6$  SN-36 cells into C3H mice causes tumor death in about 50 percent of the animals. The cells either grow in an ascites form or a solid tumor, and transitory appearance of many cells in the peripheral blood is sometimes observed in smears. However, such cells do not tend to increase and most of them finally disappear. In other words, there is a greater tendency to form a solid tumor or to form ascites than to become leukemic.

These two transplantations are between different species or different strains, and natural resistance seems to play a decisive role.

(c) In the transplantation in dd mice, difference in the growth form occurs according to pretreatment. Transplantation of Yoshida sarcoma cells into dd mice results in multiplication for a time, but the cells die out after about 1 week. Transplantation of SN-36 cells into dd mice cured of Yoshida sarcoma results in the decrease of death from tumor to about 70 percent of the animals and leukemic growth decreases to one half of those observed in nonpretreated dd mice. Here, the role of acquired resistance, besides the inherent factor, cannot be disregarded.

(d) In the transplantation into heterologous C3H mice, there is no formation of leukemia, but either X irradiation or injection of Nitromin (nitrogen mustard *N*-oxide hydrochloride) before and after transplantation to decrease the natural resistance of the host often results in the development of leukemic growth. In either case, SN-36 cells show the progress of leukemic disease when conditions for their growth are suitable and tend to undergo solid tumor growth with a longer survival time when the

condition is adverse. This can take place by the change of natural or acquired resistance, and might be called a benign change of growth. But this does not mean that there has been an irreversible change in the nature of the cells themselves.

### *Infiltration of the liver*

As has been shown, there were two kinds of infiltration in the liver at the time of death from tumor. It has been revealed by periodical observations that the infiltration of the liver first starts at the Glisson's capsule and gradually spreads to the sinusoid. Consequently, the form of disease would differ in the same animal according to the period. It is also likely that the two types would transfer mutually. To prove this point, the following experiment was carried out.

Liver lobectomy was carried out twice, 3 and 5 days after the transplantation, or once after 4 days, and the tissue was examined. This was compared with the liver tissue from the same animal after death. Infiltration seen in these tissues was: limited type (L) when there was infiltration chiefly in the Glisson's capsule and only a slight or negligible infiltration in the lobule, and diffuse type (M) when there was diffuse infiltration in the Glisson's capsule and parenchyma. There was also a type (Z) in which fairly limited infiltration foci were present in the Glisson's capsule and medium infiltration in the lobule.

Of the 18 animals used for this experiment, all the specimens examined 3 and 4 days after transplantation showed limited infiltration, but one showed M type in the excised sample from the liver taken for the second time 5 days after transplantation. The tissues examined after death of the animals due to tumor showed the limited type in 8 cases, intermediate type in 4, and complete transition to the diffuse type in 6. Infiltration foci in the Glisson's capsule were still small 3 days after transplantation but larger 4 or 5 days later. This seemed to suggest that it is not the small limited type that changes into diffuse type during development but that the cells migrate from the medium-sized limited infiltration foci formed once, and the foci themselves decrease in size. This seemed to indicate also that the infiltration type is not always constant but undergoes change during the progress of the disease.

### *Relationship between types of liver infiltration and number of tumor cells in the blood*

When there are many tumor cells in the blood, numerous cell emboli must be formed in the liver lobule and diffuse infiltration is probable. According to quantitative treatment of the immediate passage of tumor cells through various organs, passage of cells through the blood vessels of the liver and lung is far smaller than that through other parenchymatous organs, and over 90 percent of the cells is detained. It seems natural, therefore, that such diffuse infiltration is formed selectively in the liver and lung. This was proved by the following observation.

Infiltration into the liver was as described and the relationship between the infiltration type and number of tumor cells in the blood of the same animal was examined. The number of tumor cells counted nearest to the time of death was taken as the effective value. Of the 88 animals examined, 6 showed  $10^5$  cells per  $\text{mm}^3$  of tumor cells in the blood, and all the animals showed a type of diffuse infiltration in the liver. When the number of tumor cells was less than  $10^4$  cells per  $\text{mm}^3$ , 25 of 27 animals had limited infiltration, with one of the remaining two showing medium infiltration. With the number of tumor cells between these two limits, 22 of 41 animals with less than  $4 \times 10^4$  tumor cells in the blood had limited type, and 10 of 14 with more than that but less than  $10^5$  tumor cells had diffuse infiltration (table 4). Thus, animals having a large number of tumor cells in the blood had diffuse infiltration. In other words, tumor cells were evenly distributed throughout the whole body in such animals.

The same relationship existed between the number of tumor cells in the blood and two types of infiltration in the lung.

The number of tumor cells in blood and types of infiltration in the liver or lung are influenced by a very slight change in the host's resistance, and the facile transition of such infiltration, according to the period of the progress of disease, may be a characteristic of leukemic infiltration.

TABLE 4.—Correlation between number of tumor cells in the circulating blood and type of liver infiltration

Number of tumor cells in circulating blood/ $\text{mm}^3$	Number of cases:		
	Localized in Glisson's capsule	Mixed form	Diffuse in acinus
$>10^5$	0	0	6
$>4 \times 10^4$	4	0	10
$>10^4$	22	14	5
$<10^4$	25	1	1
Total	51	15	22

## LEUKEMOID REACTION INDUCED BY LEUKEMIC GROWTH

Leukemoid reaction occurs in various inflammations and malignant tumors, according to studies of human autopsies (43, 44). Leukemoid reaction in malignant tumor is due to the liberation of immature hematopoietic cells into the blood by both hyperplasia of hematopoietic tissue induced by some proliferative stimulation and destruction of its structure by tumor metastasis. In carcinoma and sarcoma, the number of tumor cells invading the blood is very small and they can be distinguished from immature hematopoietic cells quite easily because of their characteristic form so that there is no difficulty in the study of leukemoid reaction. If this reaction were induced by carcinoma or sarcoma, the same reaction

would occur in leukemia because the disease is due to similar tumor growth. Leukemic cells have special affinity to bone marrow and lymph nodes, more so than carcinoma or sarcoma cells, and leukemoid reaction should appear more strongly than that in tissue tumors. In human disease, however, it is virtually impossible to know whether the cells appearing in the leukemoid reaction are normal, immature cells, neoplastic leukemia cells, or mature tumor cells formed by their differentiation. SN-36 cells never show up in maturation so that their discrimination from immature cells of blood tissue is comparatively easy. By the use of this idea, a series of examinations is being made for reactive cells in the peripheral blood and a brief outline of this work is given.

A total of 79 dd mice were transplanted with SN-36 cells into the peritoneum and the number of white cells in the peripheral blood was calculated every day, before and after transplantation, until death of the animals. A complete examination of the blood was carried out on 46 animals. In addition, 10 dd and 7 C3H mice given intravenous transplantation of SN-36 cells were examined. Examination of peripheral blood was also made in 25 C3H mice that had been given intra-abdominal (intraperitoneal) injection of 50 mg per kg of Nitromin before, and 2 days after, the intravenous transplantation. Nitromin had no inhibitive effect on the growth of this tumor.

The number of white cells decreased the day after transplantation but subsequent variation in their number was not constant. This form of variation was classified in three types (table 5): 1) There was no marked change in the number of white cells before transplantation of SN-36 cells, nor did they tend to decrease. Twenty-four of 79 dd mice with intra-abdominal transplantation and C3H mice without treatment showed this type of variation. 2) White cells gradually decreased for 4 to 5 days after transplantation, followed by rapid increase. Ten dd mice given intravenous transplantation in the tail vein and 44 of 79 given intra-abdominal transplantation showed this type of variation. 3) White cells gradually increased, with some fluctuation in the number. The increase, however, was not more than 3 times the original number in any animal before death.

When white cells increased rapidly, tumor cells appeared 3 to 4 days after transplantation and the cells increased rapidly thereafter. Increase

TABLE 5.—Changes in the number of white blood cells in peripheral blood of animals transplanted with SN-36 cells

Change in white blood count	Intra-peritoneal transplantation (dd mice)	Intravenous transplantation (dd mice)	(C3H mice)
No marked increase	24/79*	0/10	6/7
Gradual increase	11/79	0/10	1/7
Marked increase	44/79	10/10	0/7

\*Number of animals showing change/No. of mice examined.

of tumor cells was accompanied by reactive leukocytosis, including immature type of white cells. Leukocytosis was over 5 times higher than before transplantation in the most affected animals. Twenty of 31 animals given intra-abdominal transplantation showed the reaction of granulocyte system, while 11 had a marked increase of both granulocytes and lymphocytes. Eight of 10 animals given intravenous transplantation had increased granulocytes and lymphocytes, and the remaining 2 showed a marked increase of only the lymphocytes.

In the first group showing almost no increase of white cells, a few tumor cells appeared and disappeared on different days and there was no marked increase of these cells. These animals had only a slight degree of leukemic organ infiltration.

In the untreated C3H mice with transplantation of SN-36 cells, reactive leukocytosis was not observed, though there was a temporary appearance of tumor cells which disappeared at death. In 2 of the C3H mice treated with Nitromin, more than 5,000 per mm<sup>3</sup> tumor cells were found in the peripheral blood, while tumor cells appeared during the terminal stage in 23 other animals. Including the former 2 animals, a total of 8 animals showed a marked reactive leukocytosis. Such evidence indicates that a leukemoid reaction is caused by the growth of a leukemic tumor.

Histological findings corresponding to these changes of white cells in the peripheral blood were examined. Animals given intravenous transplantation had an accumulation of tumor cells in the germinal center of the cortical follicle of lymph nodes (fig. 33). Later, the tumor filled the follicle, replacing the previously existing cells, and lymphocytes or intermingled lymphocytes and tumor cells wandered into the medullary sinus after 3 to 4 days (fig. 34). In the animals given intravenous and intra-abdominal transplantation, tumor cell emboli were found in the arterioles of the bone marrow, and this was followed by scattered lesions of hemorrhage and necrosis. These changes were observed immediately after transplantation to approximately 24 hours later. Tumor cells were scattered in the medullary cord of the bone marrow or formed a severe infiltration in 4 to 5 days. The venous sinus became markedly enlarged and the wall of the sinus, in which a large number of white cells and tumor cells were floating, was destroyed. These findings seemed sufficient histological evidence that a leukemoid reaction accompanied growth of a tumor.

## LEUKEMIC INFILTRATION INTO SPECIFIC ORGANS

### Infiltration Into Granuloma

It has been stated that infiltration was not formed in the brain substance, subcutaneous tissue, or muscles when SN-36 cells were transplanted intravenously. If the tumor cells are transplanted directly into these

TABLE 6.—Formation of leukemic infiltration into granulation tissue around foreign substance inserted into the brain of mice following intravenous transplantation of SN-36 cells

Interval from insertion to transplantation (days)	Kind of substances inserted							
	Paraffin	Liver*	Muscle†	Bone†	Liver‡	Spleen‡	Lung‡	Total
3			2/4§	2/4	2/4	1/3	1/4	8/19
5	3/3	3/3	4/5	0/4	1/2	2/3	0/2	13/22
7	3/3	2/2	3/4	3/4	2/3	3/4	3/4	19/24
10	3/3	3/3	5/5	1/3	1/2	2/3	0/4	15/23
20	1/5		0/6	2/3	0/5	0/4	0/2	3/25
30								0/3
45	1/6	0/2						1/8
Total	11/23	8/10	14/24	8/18	6/16	8/17	4/16	59/124

\*Fixed in ethyl alcohol.

†Fresh.

‡Fresh, in plastic tube.

§Numerator: No. of cases with leukemic infiltration of tumor cells/No. of cases examined.

sites, local growth of tumor occurs. Consequently, failure of infiltration in these sites by intravenous transplantation is possibly due to immediate passage of tumor cells through these organs transported in the blood stream. To test this hypothesis, the following experiment was carried out.

Mice of dd strain, obtained from a dealer and weighing about 20 g, were used. A small hole was drilled in the skull of 174 animals and paraffin block, alcohol-fixed liver, plastic tubes, small pieces of fresh muscle, or bone chips were inserted into the brain, and the skin was closed. One million SN-36 cells were injected intravenously into 124 animals on the 3d, 5th, 7th, 10th, 20th, and 45th day after insertion of the substances into the brain. The site of brain surgery was examined when the animal died of leukemic tumor. The remaining 50 animals were killed at the time of transplantation and granulation was examined histologically (table 6).

In 20 mice, foreign bodies, such as cinnabar, were injected subcutaneously with a large caliber needle. Three of these animals were killed 8 days later for examination of granulation and 17 others were given injections of  $10^6$  SN-36 cells in their tail vein (table 7).

TABLE 7.—Formation of subcutaneous granuloma by insertion of foreign substances and leukemic infiltration therein of SN-36 cells transplanted intravenously 8 days after the insertion

Foreign substance	Leukemic infiltration
Cinnabar	3/3*
Mixture of cinnabar and liver tissue	4/4
Mixture of rat tumor and liver tissue	4/4
Plastic	1/6

\*Number of positive cases/No. of animals examined.

TABLE 8.—Leukemic infiltration into granulation tissue induced by the fracture of femur of mice followed by intravenous transplantation of SN-36 cells

Interval from fracture to intravenous transplantation (days)	Leukemic infiltration
2	2/2*
4	2/2
6	1/1
10	1/2
15	0/2
40	0/1

\*Number of positive cases/No. of animals used.

Femoral muscle of 5 animals was crushed; 1 mouse was killed 5 days later, and 4 mice were given intravenous transplantation of SN-cells.

Fracture of the femur was produced in 16 animals and one was killed after 2, 4, 6, 10, 15, and 40 days for examination of granulation. On each of these days, 10 animals were intravenously transplanted with  $10^6$  cells and leukemic infiltration into the local site was examined histologically at death (table 8).

Results of these experiments are illustrated in tables 6, 7, and 8. Positive changes (leukemic infiltration) were seen in 59 of 124 animals in the group having brain surgery (figs. 46 and 47), in 6 of 10 animals in the fracture group, in all 4 with crushed muscle (fig. 48), in 12 of 17 animals given injections of foreign substances subcutaneously (fig. 49).

Correlation between the infiltration and number of days from insertion of foreign bodies until transplantation was as follows: In the brain group, the highest incidence was found in the interval of 7 days, with positive changes seen in 19 of 24 animals. An interval of 5 to 10 days also produced infiltration, while 3 days was less effective, and an interval of 20 days hardly produced infiltration. Condition of granulation showed conspicuous formation of new blood vessels between 5 and 10 days, and the fibrous element was formed more after 20 days, with few blood vessels.

In the fracture group, severe fibrous growth was observed on the 6th day after the fracture.

With subcutaneous injection of cinnabar, specific granuloma due to increase of phagocytes was noticed. Infiltration started around the newly formed blood vessels in this granuloma and appeared to be proliferating, as if replacing the tissue.

In the group with crushed muscle, bone fracture, and subcutaneous foreign bodies, some animals showed a tumorous growth, from the size of a rice grain to a small bean.

## SUMMARY

The histogenesis of leukemia was discussed from the viewpoint that leukemia is considered as a neoplastic entity. The exogenous agents

seemed important, as were the endogenous factors for the course of development. The process of leukemic development was discussed, based on the results obtained in the quantitative study of the thymic lymphoid cells of the AKR mice which developed thymic lymphomas. Development of leukemic infiltration was examined by use of transplantable leukemia SN-36, and the variety of morphological manifestations in leukemia, concerning either the general factors in the host or the local condition in various organs and tissues, was discussed.

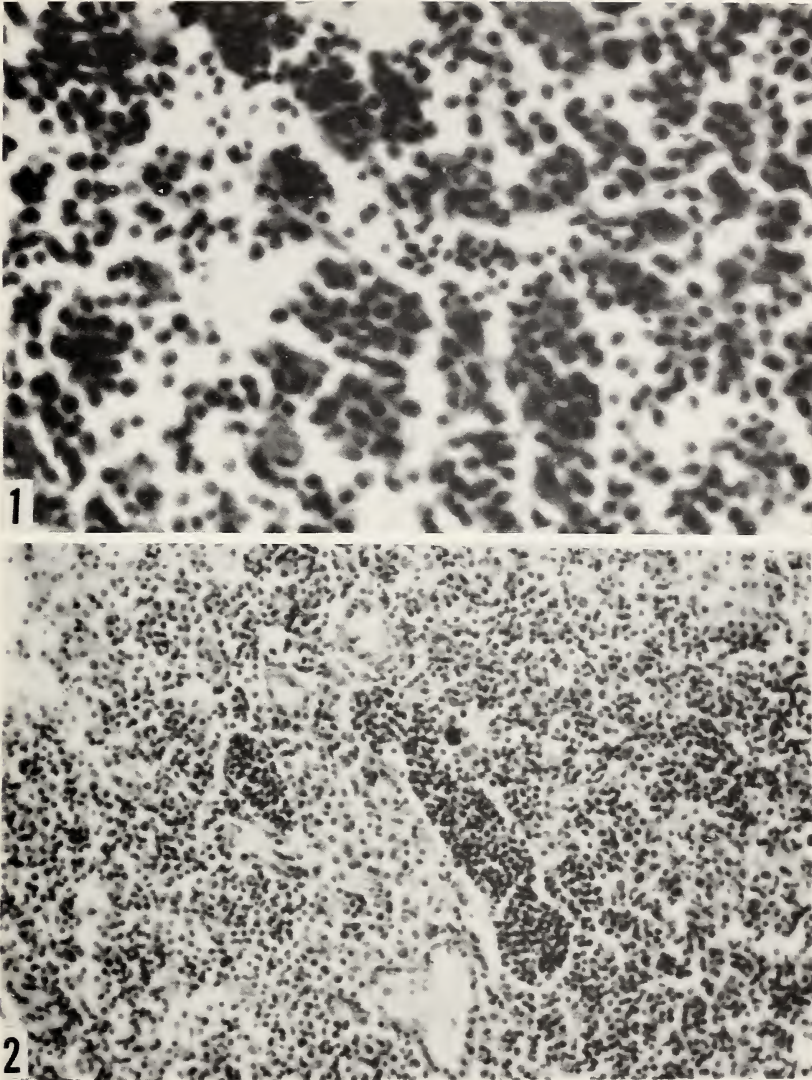
## REFERENCES

- (1) BRUES, A. M., and MARBLE, B. B.: Lymphoblastoma in mice following administration of carcinogenic tar. *Amer J Cancer* 37: 45-53, 1939.
- (2) FURTH, J., and FURTH, O. B.: Monocytic leukemia and other neoplastic diseases occurring in mice following intrasplenic injection of 1:2-benzpyrene. *Amer J Cancer* 34: 169-183, 1938.
- (3) MIDER, G. B., and MORTON, J. J.: The effect of methylcholanthrene on the latent period of lymphomatosis on dilute brown mice. *Amer J Cancer* 37: 355-363, 1939.
- (4) RASK-NIELSEN, R.: Investigations into the varying manifestations of leukaemic lesions following injections of 9:10-dimethyl-1:2-benzanthracene into different subcutaneous sites in Street mice. *Brit J Cancer* 3: 549-556, 1949.
- (5) IKEDA, Y. T., TOKUDA, Y., MORIMURA, Y. L., YASUOKA, M., and MORITA, J.: Myeloid leukemia caused by 2-acetylaminophenanthrene. II. The change in the hematopoietic organs at preleukemic stage. *Gann* 49 (Suppl): 150-151, 1959.
- (6) WATANABE, Z.: Histopathological studies on leukemogenesis using radioisotopes in animals. *Acta Path Jap* 46: 183-239, 1957.
- (7) WARABIOKA, K.: Preliminary report on carcinogenic experiment with methyl-di(2-chloroethyl)amine-N-oxide hydrochloride (Nitromin). *Gann* 44: 273-274, 1953.
- (8) GARDNER, W. U., DOUGHERTY, T. F., and WILLIAMS, W. L.: Lymphoid tumors in mice receiving steroid hormones. *Cancer Res* 4: 73-87, 1944.
- (9) KIRSCHBAUM, A., SHAPIRO, J. R., and MIXER, H. W.: Synergistic action of leukemogenic agents. *Cancer Res* 13: 262-268, 1953.
- (10) FRIEND, C.: Leukemia in adult mice caused by a transmissible agent. *Ann NY Acad Sci* 68: 522-532, 1957.
- (11) FURTH, J., and METCALF, D.: An appraisal of tumor-virus problems. *J Chronic Dis* 8: 88-112, 1958.
- (12) MOLONEY, J. B.: Biological studies on a lymphoid-leukemia virus extracted from Sarcoma 37. I. Origin and introductory investigations. *J Nat Cancer Inst* 24: 933-951, 1960.
- (13) STEWART, S. E., EDDY, B. E., and BORGESE, N.: Neoplasms in mice inoculated with a tumor agent carried in tissue culture. *J Nat Cancer Inst* 20: 1223-1243, 1958.
- (14) TAKEDA, K., and KOBAYASHI, H.: Causation of leukemia, with special reference to viruses in mouse leukemia. *Saishin-Igaku* 17: 397-406, 1962.
- (15) NAKAMURA, K.: Histopathological studies on leukemia, leukemia-like lesions, and leukemoid reaction in mice fed with azodye. *Acta Path Jap* 48: 1264-1287, 1959.
- (16) YOSHIDA, T.: Studies on an ascites (reticuloendothelial cells?) sarcoma of the rat. *J Nat Cancer Inst* 12: 947-969, 1952.

- (17) HENSHAW, P. S.: Leukemia in mice following exposure to X-rays. *Radiology* 43: 279-285, 1944.
- (18) KAPLAN, H. S.: Observations on radiation-induced lymphoid tumors of mice. *Cancer Res* 7: 141-147, 1947.
- (19) ———: Influence of thymectomy, splenectomy, and gonadectomy on incidence of radiation-induced lymphoid tumors in strain C57 black mice. *J Nat Cancer Inst* 11: 83-90, 1950.
- (20) KAPLAN, H. S., BROWN, M. B., and PAULL, J.: Influence of postirradiation thymectomy and of thymic implants on lymphoid tumor incidence in C57BL mice. *Cancer Res* 13: 677-680, 1953.
- (21) LAW, L. W., and MILLER, J. H.: Observations on the effect of thymectomy on spontaneous leukemias in mice of the high-leukemic strains, RIL and C58. *J Nat Cancer Inst* 11: 253-262, 1950.
- (22) ———: The influence of thymectomy on the incidence of carcinogen-induced leukemia in strain DBA mice. *J Nat Cancer Inst* 11: 425-437, 1950.
- (23) NISHIZUKA, Y.: Lymphatic leukemia and lymphosarcoma induced experimentally in mice. *J Jap Ass Hematol* 18: 517-528, 1955.
- (24) McENDY, D. P., BOON, M. C., and FURTH, J.: On the role of thymus, spleen, and gonads in the development of leukemia in a high-leukemia stock of mice. *Cancer Res* 4: 377-383, 1944.
- (25) METCALF, D.: The thymic lymphocytosis-stimulating factor. *Ann NY Acad Sci* 73: 113-119, 1958.
- (26) METCALF, D., and ISHIDATE, M.: Periodic acid-Schiff positive giant cells in the mouse thymus cortex. *Nature (London)* 191: 305, 1961.
- (27) NAKAMURA, K., and METCALF, D.: Quantitative cytological studies on thymic lymphoid cells in normal, preleukaemic and leukaemic mice. *Brit J Cancer* 15: 306-315, 1961.
- (28) CRONKITE, E. P., FLEIDNER, T. M., BOND, V. P., RUBINI, J. R., BREECHER, G., and QUASTER, H.: Dynamics of hemopoietic proliferation in man and mice studied by  $H^3$ -thymidine incorporation into DNA. *Ann NY Acad Sci* 77: 803-820, 1959.
- (29) SATO, H.: Intraperitoneal transplantation of the Yoshida ascites sarcoma and ascites hepatoma to various American strains of rats. *J Nat Cancer Inst* 15: 1367-1378, 1955.
- (30) METCALF, D., and NAKAMURA, K.: Transplantation bioassay of thymuses from preleukaemic AKR mice for the presence of leukaemic cells. *Brit J Cancer* 15: 316-321, 1961.
- (31) BERENBLUM, I.: A speculative review: The probable nature of promoting action and its significance in the understanding of the mechanism of carcinogenesis. *Cancer Res* 14: 471-477, 1954.
- (32) YOSHIDA, T.: Yoshida sarcoma. Tokyo, Nara-Shobo, 1948.
- (33) NAKAMURA, K., and MUNAKATA, H.: Unpublished data.
- (34) HONJO, O., TAGASHIRA, Y., KOBARA, Y., MIYAKE, T., and AMANO, J.: On the leukemia-like lesions of animals by transplantation with Yoshida sarcoma. II. Intramedullary transplantation. *Gann* 42: 151-153, 1951.
- (35) USUBUCHI, I., OBOSHI, S., IIDA, T., and KOZEKI, T.: Studies on a new ascites sarcoma (Hirosaki strain) resembling Yoshida sarcoma. *Gann* 63: 130-132, 1952.
- (36) USUBUCHI, I., SUGAWARA, M., and YOSHIDA, J.: Studies on the cross immunity between heterologous tumor cells. *Gann* 51 (Suppl): 263, 1960.
- (37) NAKAMURA, K.: A new strain of transplantable lymphosarcoma (lymphatic leukemia?) in rat. *Acta Path Jap* 42: 398-401, 1953.
- (38) ———: A new transplantable leukemic lymphosarcoma of the rat. *Gann* 45: 374-378, 1954.
- (39) ———: A new transplant strain of leukemic ascites tumor of mice. *Gann* 47: 561-565, 1956.

- (40) NAKAMURA, K., and MUNAKATA, H.: Unpublished data.
- (41) MUNAKATA, H.: Studies on metastasis. XII. The fate of cancer cells in the circulating blood. Fukushima Med J 11: 1281-1285, 1961.
- (42) NAKAMARA, K.: Infiltrating findings of transplanted leukemia (lymphatic) SN-36 in the various organs. Acta Path Jap 46: 276-277, 1957.
- (43) KITAMURA, S.: Pathology of leukemoid reaction with special reference to its origin. J Jap Ass Hematol 22: (Suppl) 740-760, 1959.
- (44) MIYAKE, H.: Pathology of leukemoid reaction. J Jap Ass Hematol 14 (Suppl): 310-338, 1950.





FIGURES 1 THROUGH 10: HISTOPATHOGENESIS OF AZO-DYE INDUCED LEUKEMIA.

FIGURE 1.—Lymph node of a mouse at 18th day of dye feeding, showing many lymphocytes in the medullary sinuses.

FIGURE 2.—Lymph node at 24th day. Two masses of lymphocytes are densely compacted in the medullary sinuses, being encircled with elongated endothelial cells. Appears to be congestion of cells shed from the follicle.

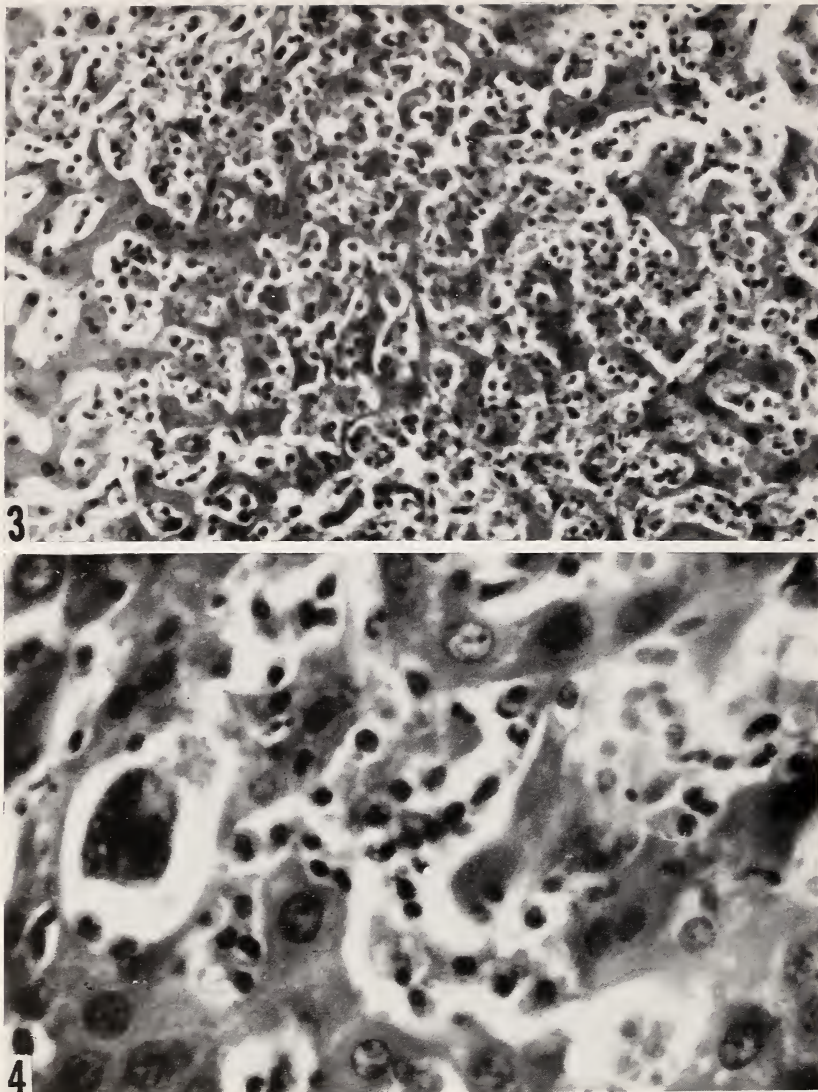
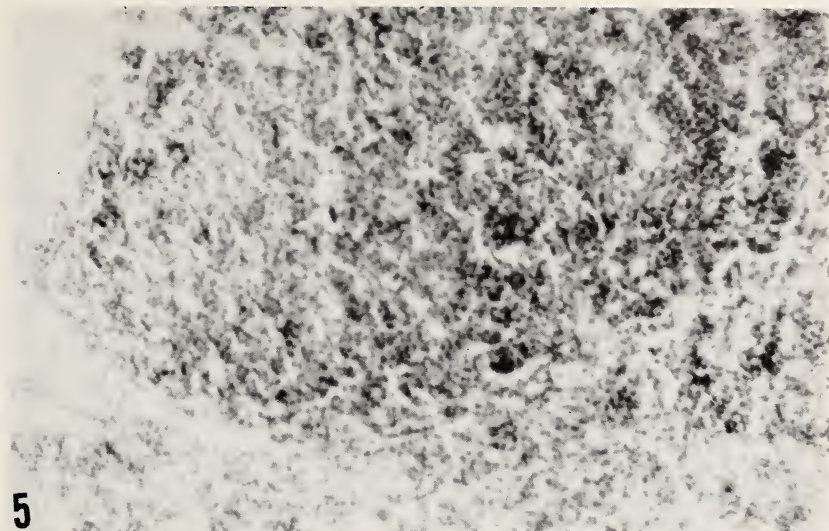
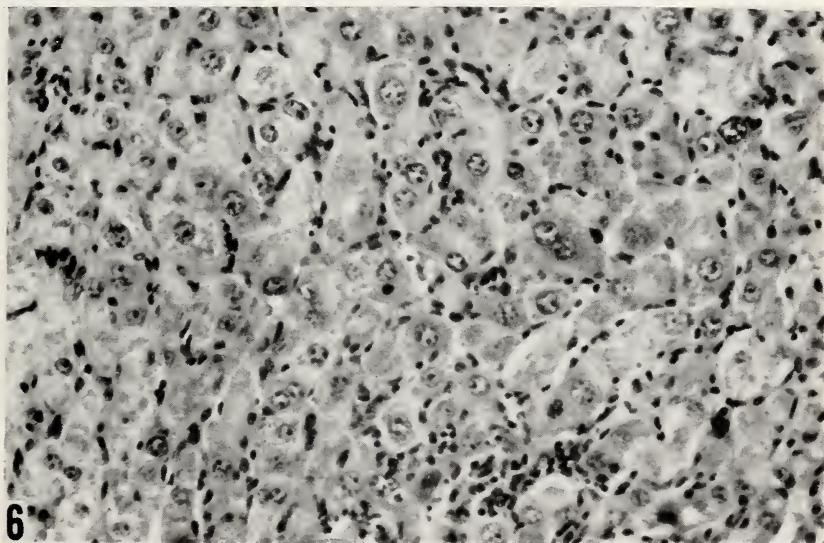


FIGURE 3.—Changes in the liver at 12th day, but not from leukemic infiltration. There are no proliferative changes anywhere else in the body. Most of the cells in sinusoid are matured monocytes, neutrophils, and lymphocytes.

FIGURE 4.—High-power magnification of changes similar to those in figure 3, with oil-immersion lense. Note giant cell in sinusoid, which originated in the spleen. These cells in sinusoid probably are temporary migrants from the lymph nodes and spleen, whose cell structure is destroyed by the action of the dye—possibly a leukemoid reaction.



5



6

FIGURE 5.—“Leukemia-like changes” in lymph node of a mouse at 201st day of *o*-amino-azo-toluene feeding. Hyperplasia of lymphoid cells and enlargement of cortical follicle are prominent.

FIGURE 6.—“Leukemia-like changes” in the liver at 298th day. Hyperplasia and metamorphosis of endothelial cells in the sinusoid make the changes like leukemia. This could be a transitional form to reticular-type leukemia.

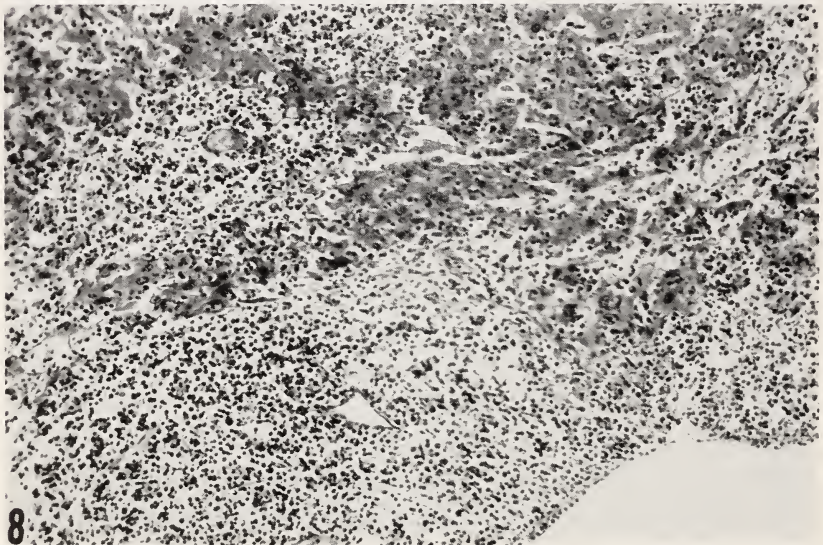
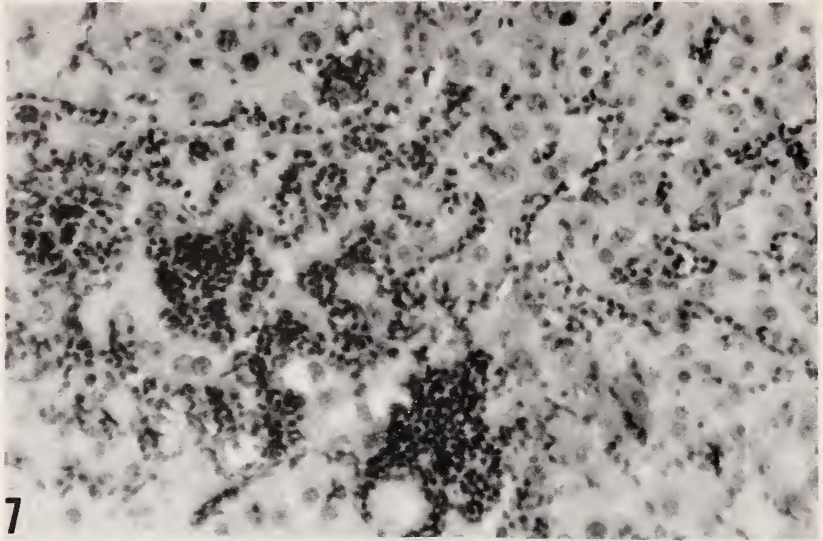


FIGURE 7.—Reticular-type leukemia invading liver. *Note* formation of masses in the sinusoid by active proliferation of the cells, as in figure 6.

FIGURE 8.—The liver after induction of lymphoid leukemia.

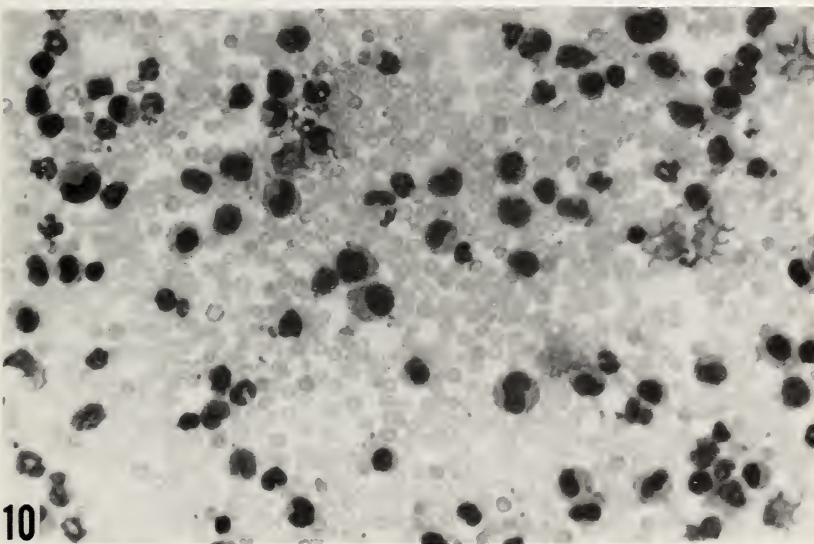
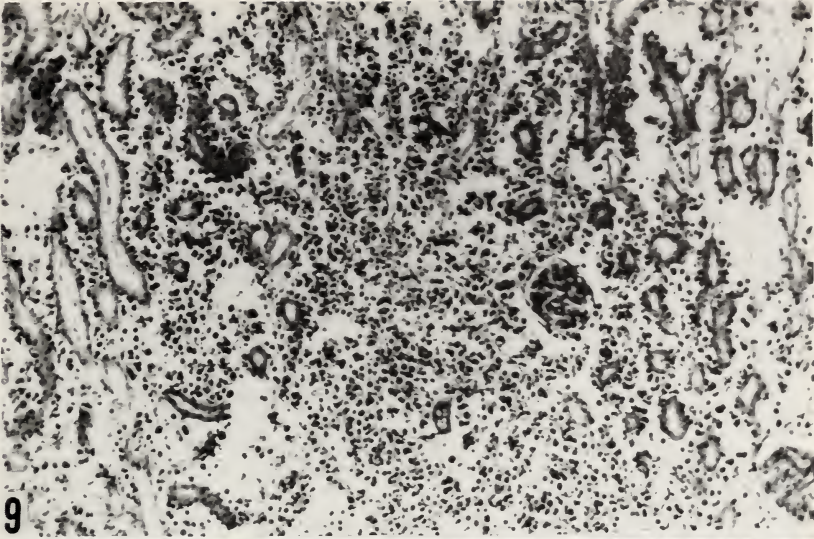
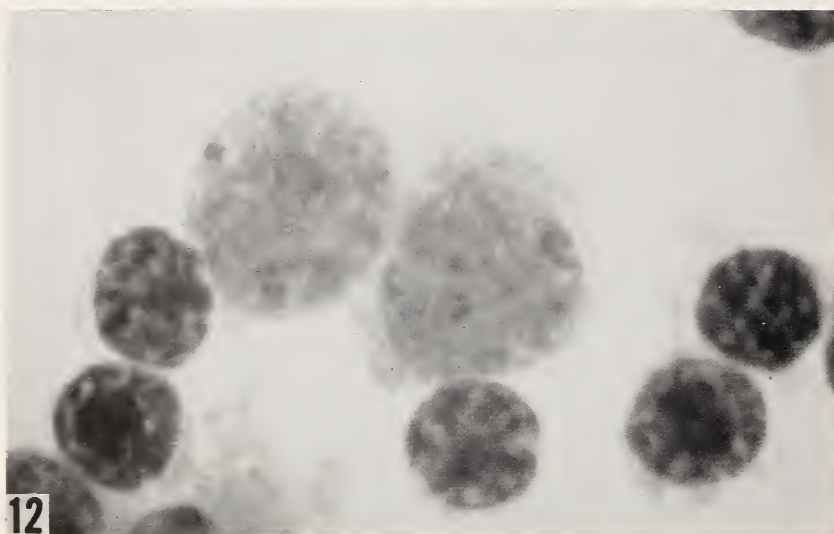
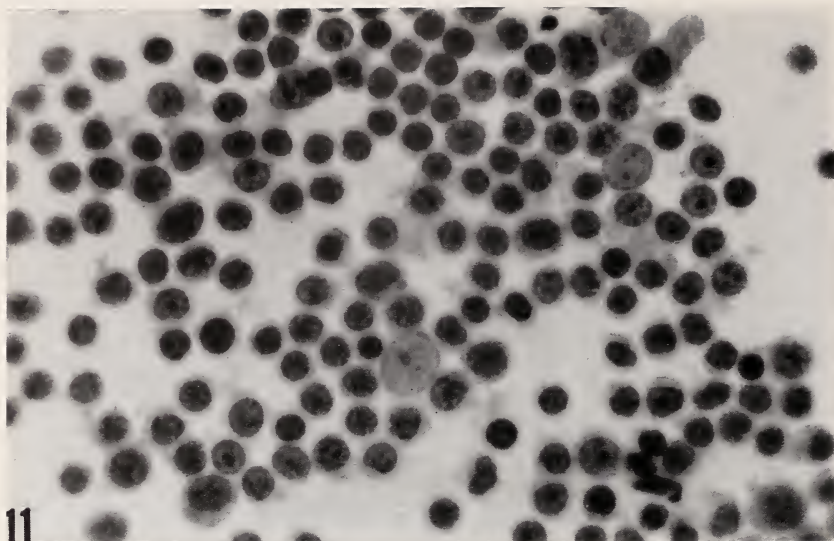


FIGURE 9.—Lesion in a kidney of same animal shown in figure 8.

FIGURE 10.—Leukemia cells in the peripheral blood of another animal. Cells resemble large lymphocytes and there are no oxidase-positive granules.



## FIGURES 11 THROUGH 17: THYMUS CYTOLOGY.

FIGURE 11.—Lymphoid cells of the thymus in wet preparation. *Note* absence of damaged cells. A large and two medium lymphocytes are seen.

FIGURE 12.—High-power magnification with oil-immersion lense. Two cells (*center*) should be classified as large lymphocytes, because of the structure of the nucleus with its tenuous chromatin network and not because of their size.

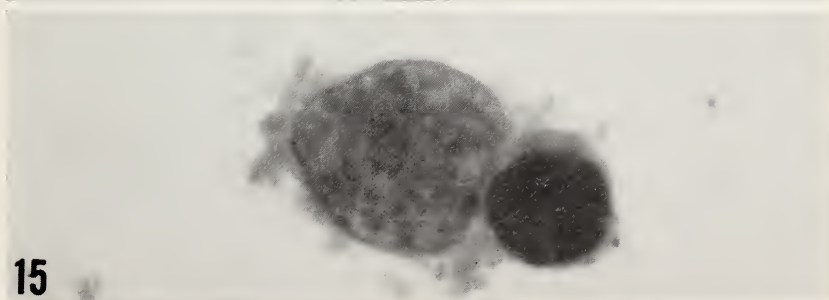
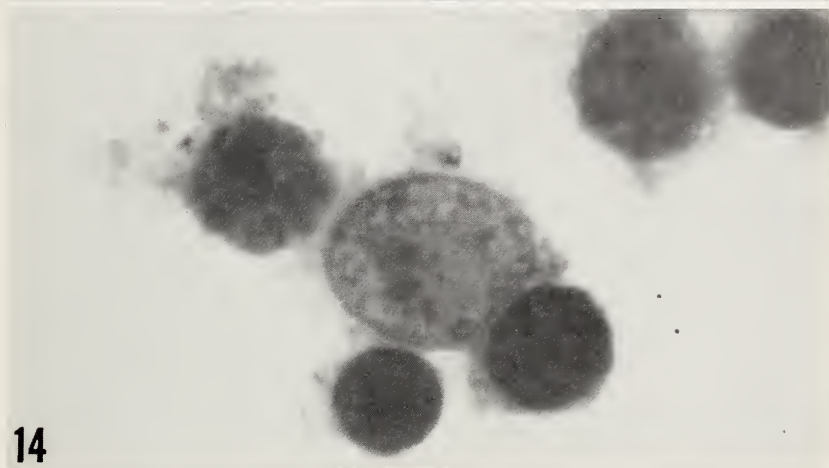
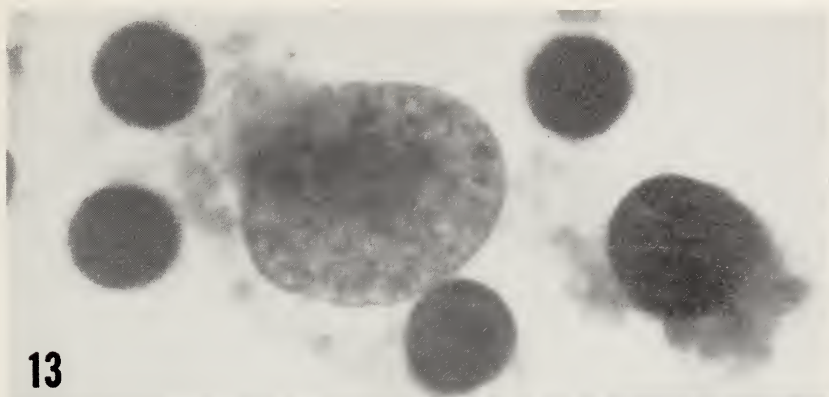


FIGURE 13.—Large lymphocyte, having fine network without any chromatin clump.

FIGURE 14.—Medium lymphocyte. Chromatin network is still fine but a few chromatin clumps are visible.

FIGURE 15.—Medium lymphocyte. Coarse network with clumps.

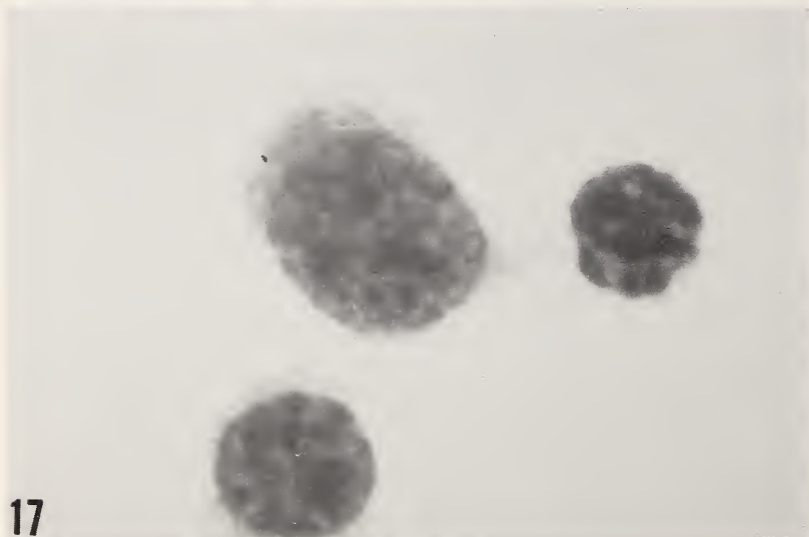
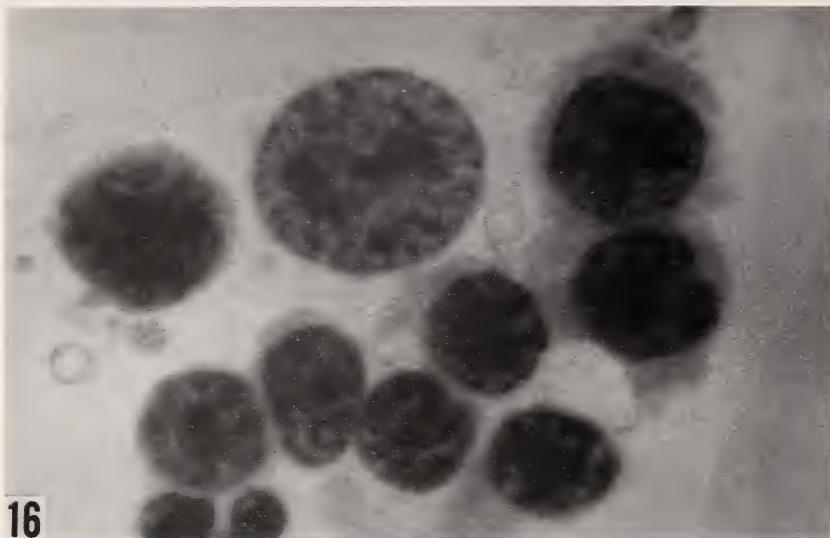
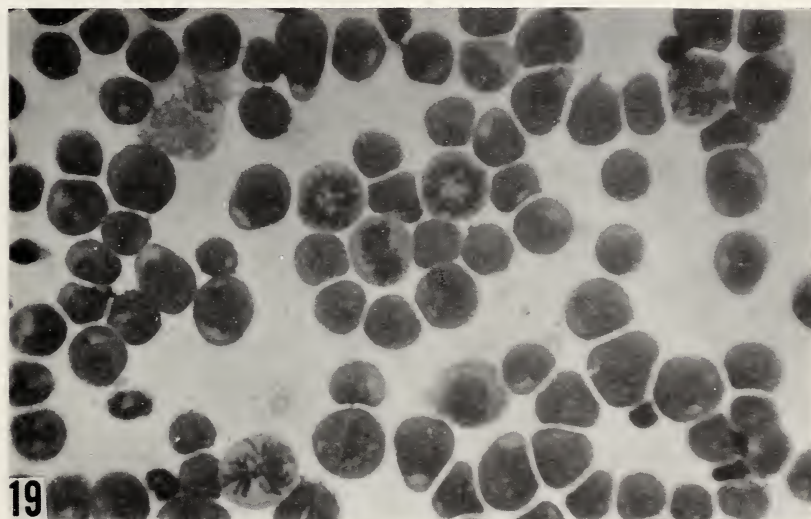
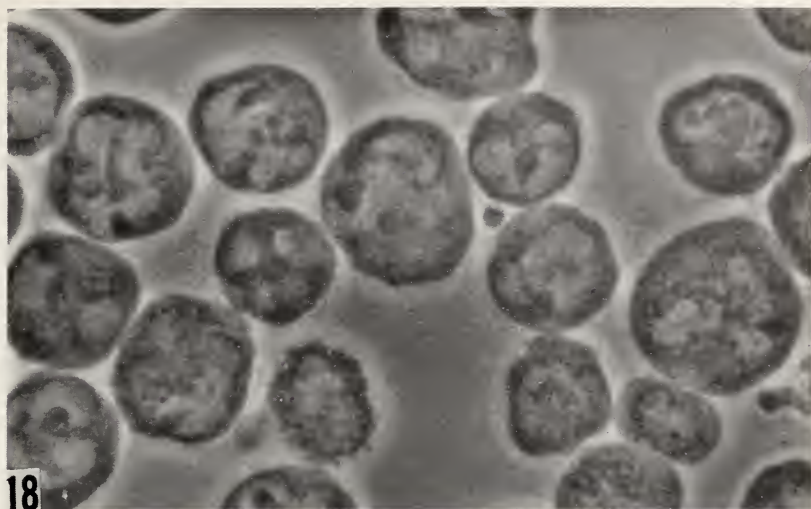


FIGURE 16.—Largest cell is medium lymphocyte with large chromatin clumps but relatively fine network. Other cells are small lymphocytes. *Note* their rough, coarse network with chromatin clumps.

FIGURE 17.—Small lymphocytes. *Note* comparatively fine network structure in one.



FIGURES 18 THROUGH 45: TRANSPLANT STRAIN OF THE LEUKEMIC ASCITES TUMOR SN-36.

FIGURE 18.—Living cells of leukemic ascites tumor SN-36 in ascites, under phase-contrast microscope. Lobulation of the nuclei, predominant nucleoli, and comparatively scanty cytoplasm with abundant mitochondria are clearly seen.

FIGURE 19.—Smear preparation of the tumor ascites by Wright-Giemsa stain. In comparison with figure 18, the lobulation of the nuclei and predominance of the nucleoli are less clear.

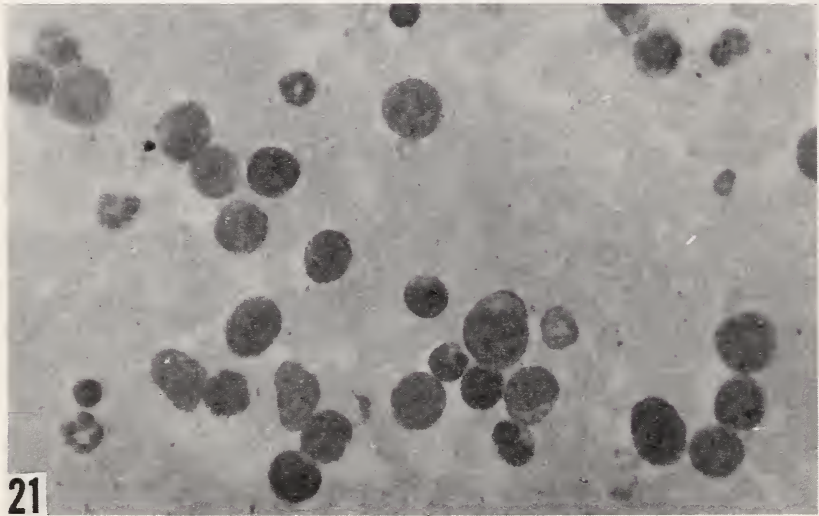
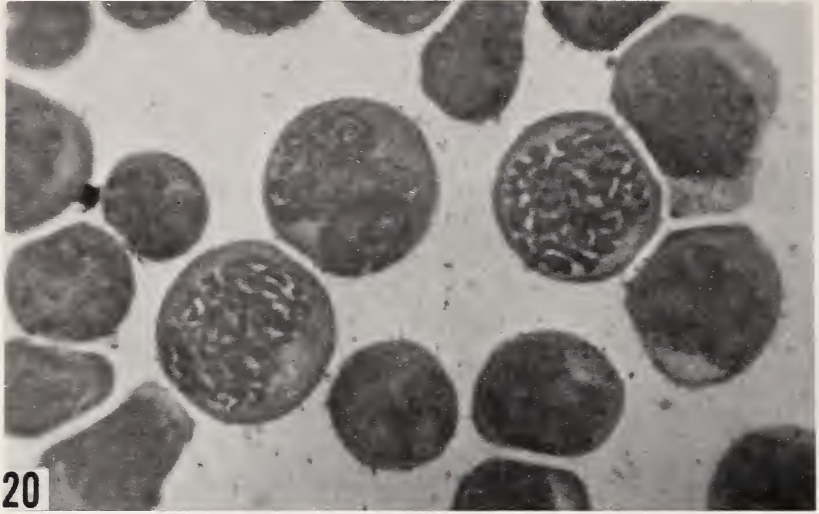
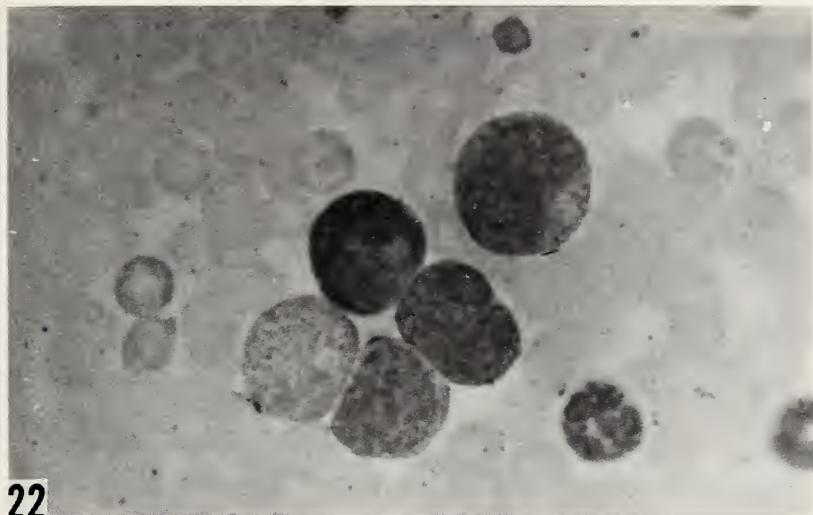
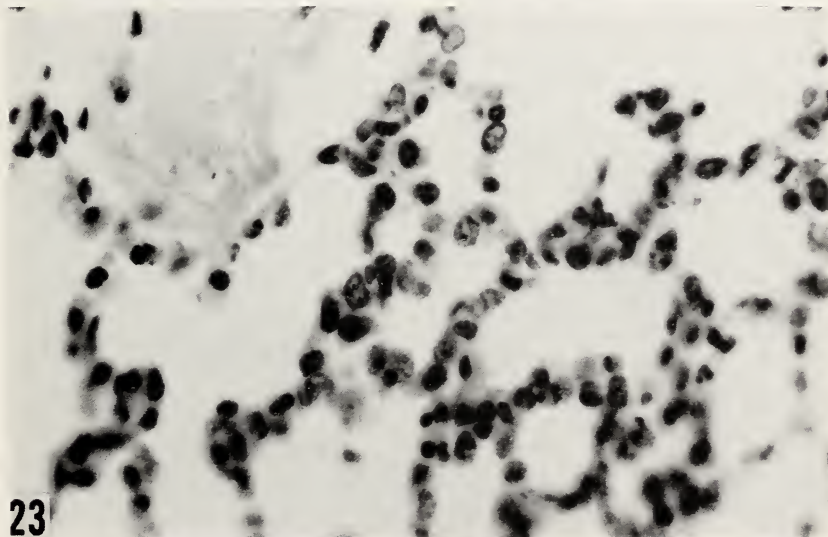


FIGURE 20.—Tumor cells under oil-immersion lense. Two cells are in process of mitosis (prophase). *Note* no stainable granules.

FIGURE 21.—Tumor cells in the peripheral blood. *Compare* with normal lymphocytes seen in the *lower center*.



22



23

FIGURE 22.—Tumor cells in peripheral blood. One tumor cell has unstainable nucleus. Degenerating tumor cells like this are often seen in blood smears but not in the ascitic fluid.

FIGURE 23.—Sectioned preparation of the lung, 10 minutes after intravenous inoculation of  $10^7$  tumor cells. Many tumor cells are in the capillaries of the alveolar septums in a state of temporary embolus. Immediate passage through the capillaries is also likely. See figure 27.

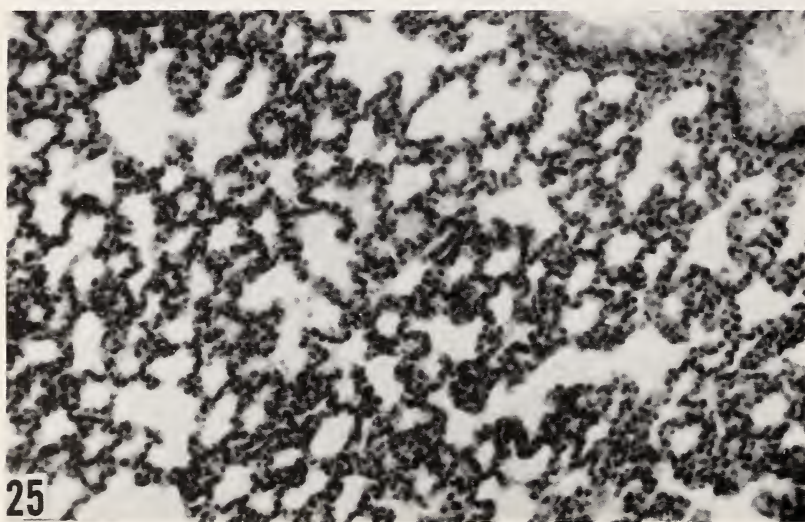
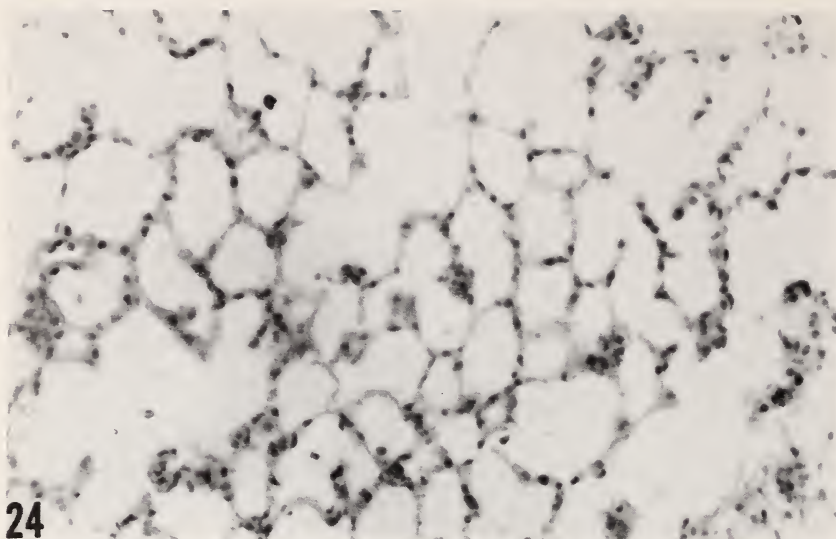


FIGURE 24.—Three hours after inoculation. Clearly, most of the embolic tumor cells have passed through capillaries and disappeared.

FIGURE 25.—One of two different manifestations of leukemic invasion into the lungs at end stage of the growth of SN-36. Diffuse and extensive invasion is clearly shown in the parenchyma without any particular localization.

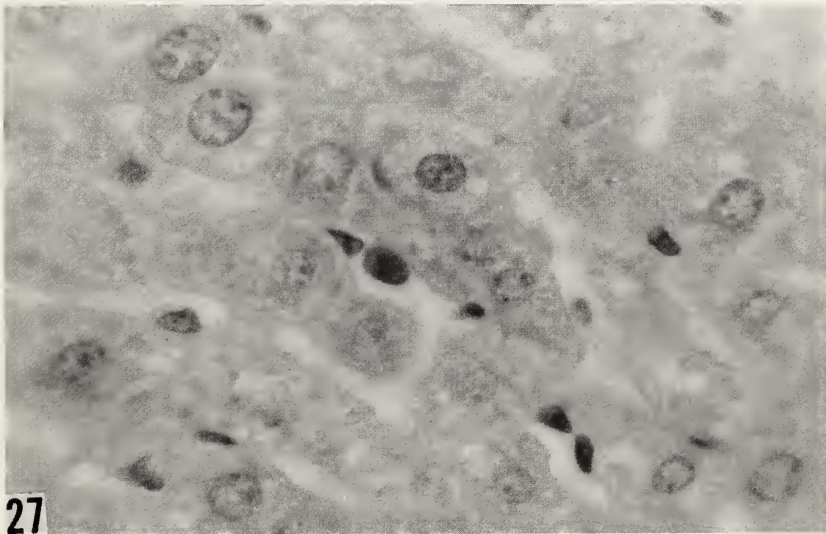
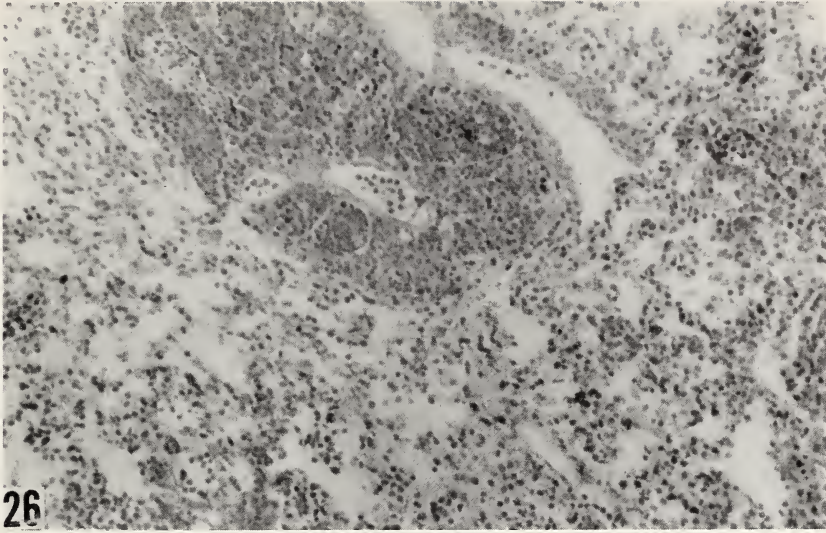


FIGURE 26.—Another manifestation of particularly intense peribronchial growth.

FIGURE 27.—Liver 10 minutes after intravenous inoculation of tumor cells. One tumor cell is visible in sinusoid.

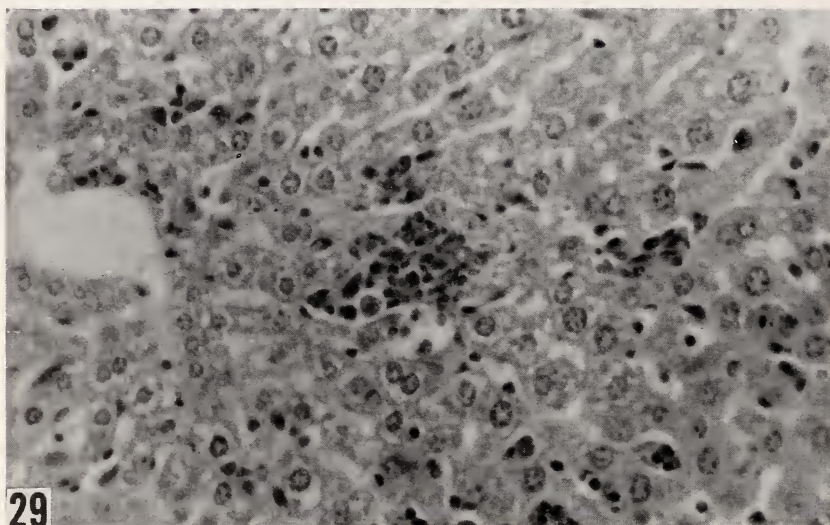
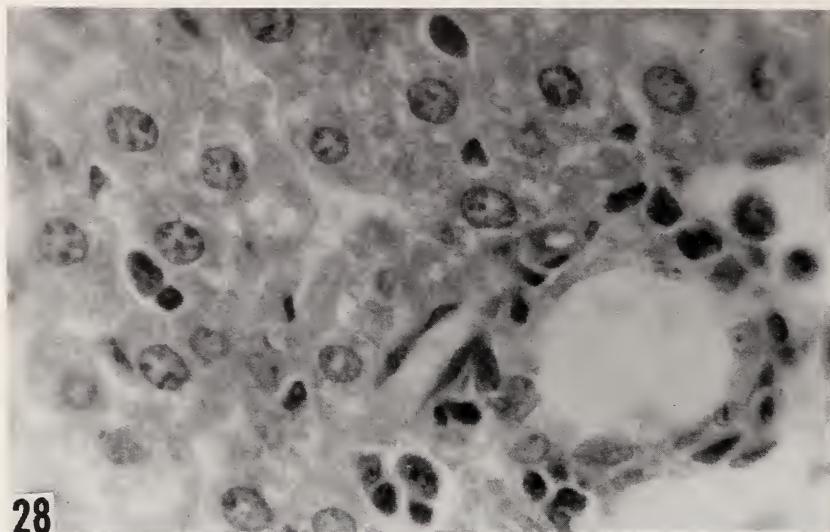


FIGURE 28.—Liver, 30 minutes after intravenous inoculation of tumor cells, showing an increase of cells.

FIGURE 29.—Liver, 3 hours after the same procedure, showing tissue reaction around the tumor cells.

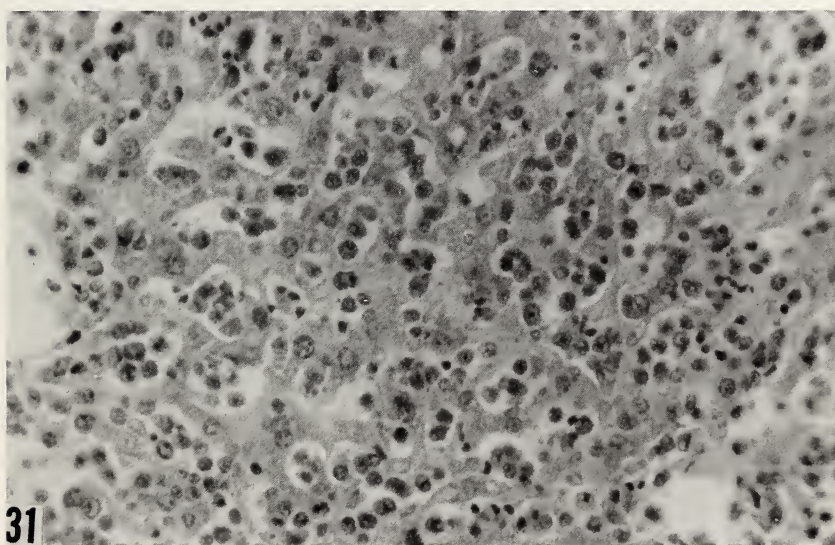
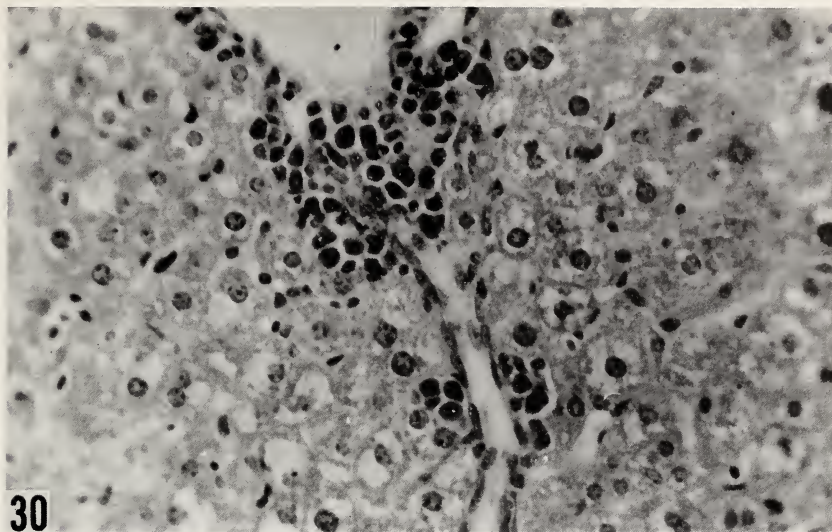


FIGURE 30.—Early stage in formation of leukemic infiltration into the liver 48 hours after inoculation of tumor cells.

FIGURE 31.—One of various manifestations of the infiltration of SN-36, showing diffuse invasion into the liver parenchyma.

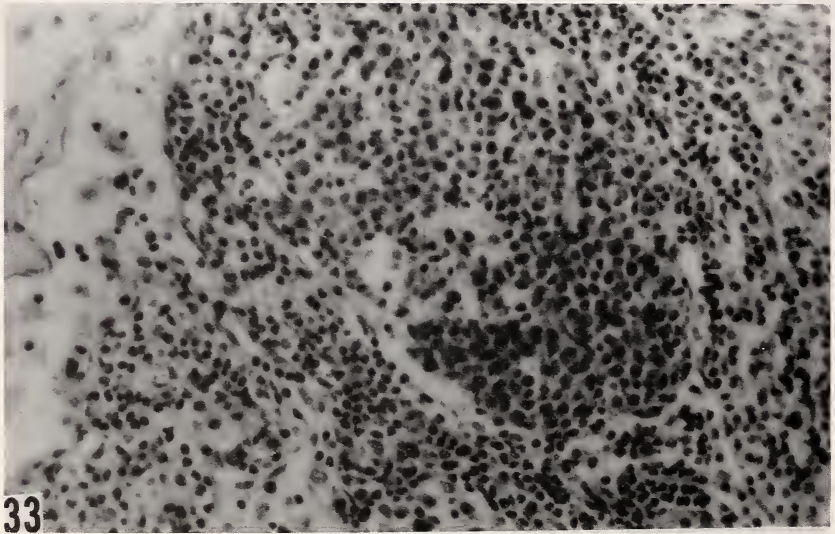
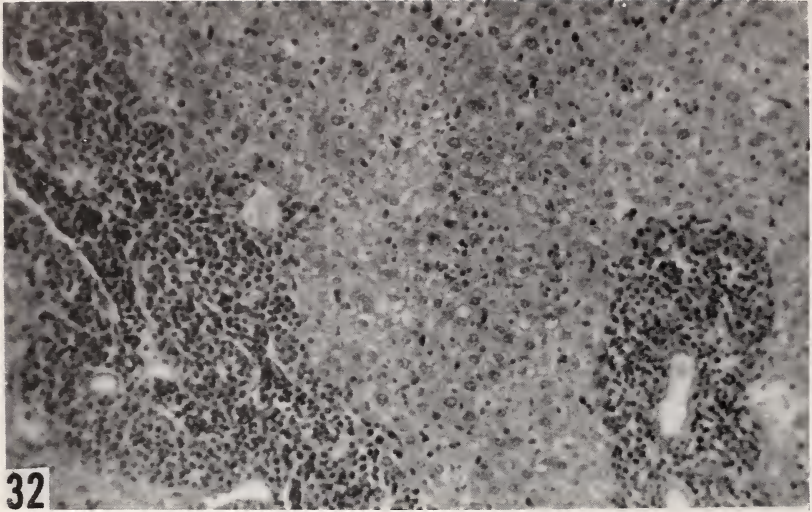


FIGURE 32.—Another type of invasion, showing localized growth of cells identical to those in figure 31. In Glisson's capsule.

FIGURE 33.—Lymph node, 48 hours after the same procedure, showing a small mass of tumor cells in *center* of the cortical follicle.

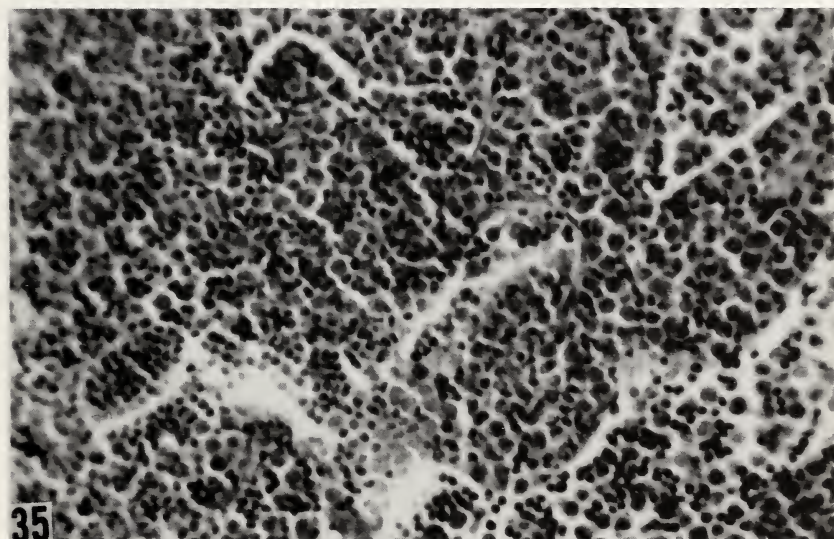
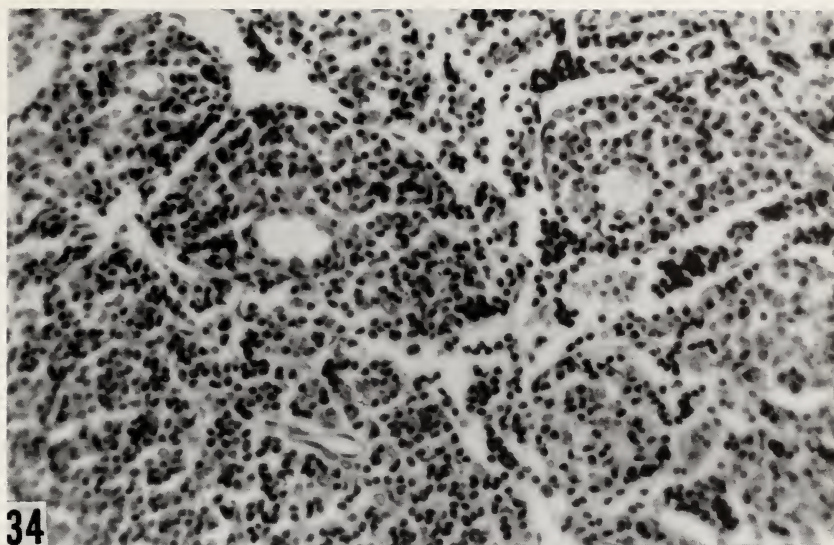


FIGURE 34.—Seventy-two hours after inoculation of tumor cells. Invasion of tumor cells and diminishing small lymphocytes are clearly seen in the medullary cord. But, many small, intensely stained lymphocytes are visible in the medullary sinus.

FIGURE 35.—Lymph node, 4 days after inoculation, showing tumor cells and lymphocytes in the sinus. Few tumor cells were found in the peripheral blood at this time.

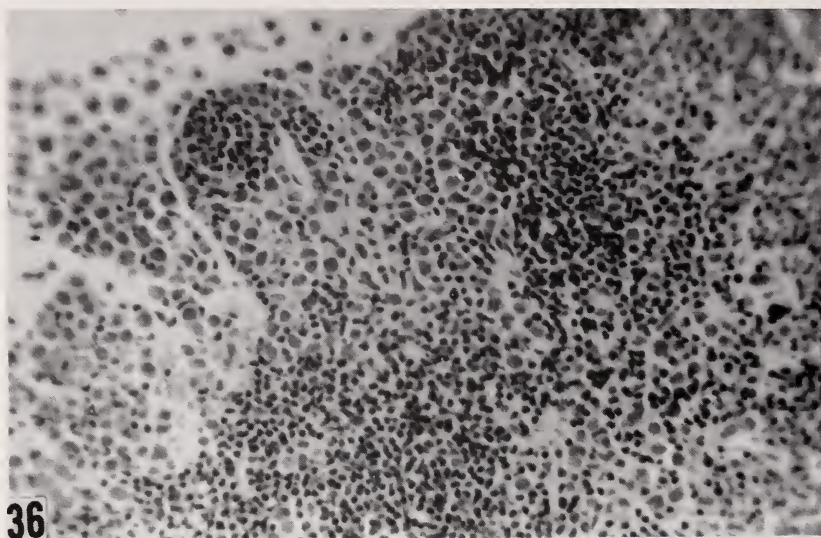


FIGURE 36.—A regional lymph node 48 hours after subcutaneous transplantation of tumor cells. Tumor cells are mostly found in subcapsular sinus (interfollicles) but less in the follicles.

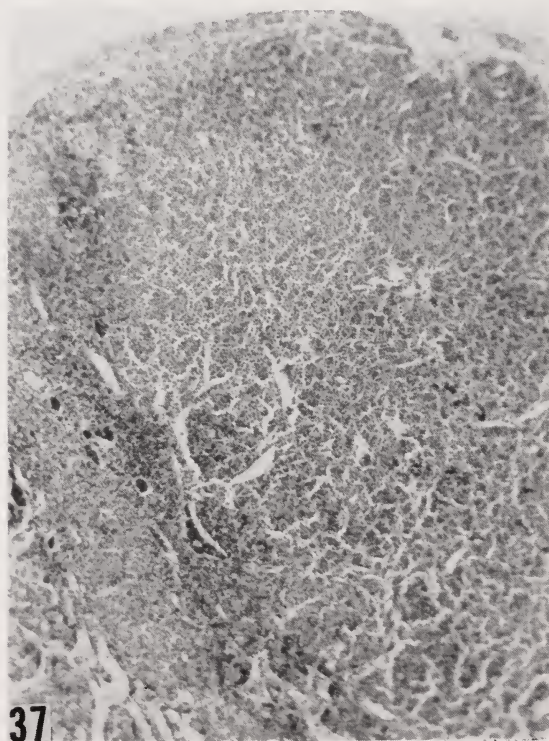
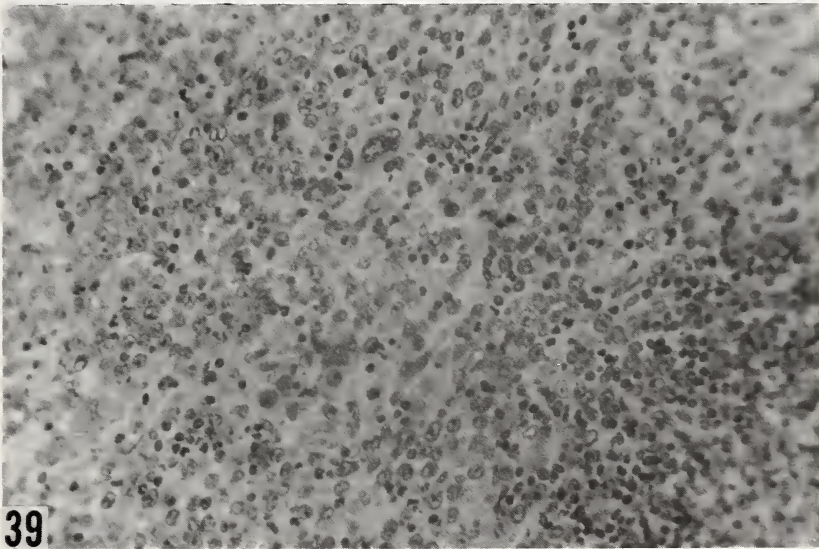
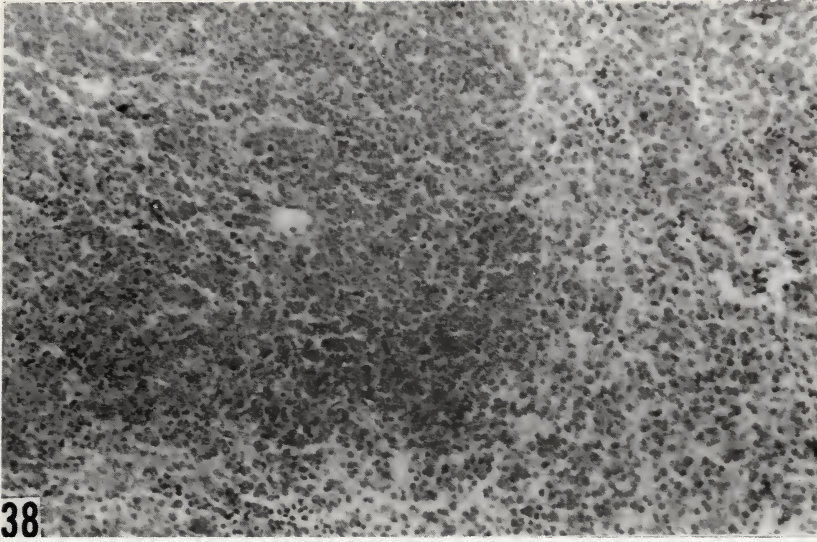


FIGURE 37.—Final stage of the lymph node infiltration.



DIFFERENT MANIFESTATIONS OF LEUKEMIC INFILTRATION INTO  
THE SPLEEN OF SN-36.

FIGURE 38.—The follicular type. Follicle of the spleen is enlarged and replaced by tumor cells. This part is seen as denser mass of tumor cells with central artery.

FIGURE 39.—Red pulp of the spleen clearly invaded more intensely than the follicle seen at *corner*. Note splenic megakaryocytes remain.

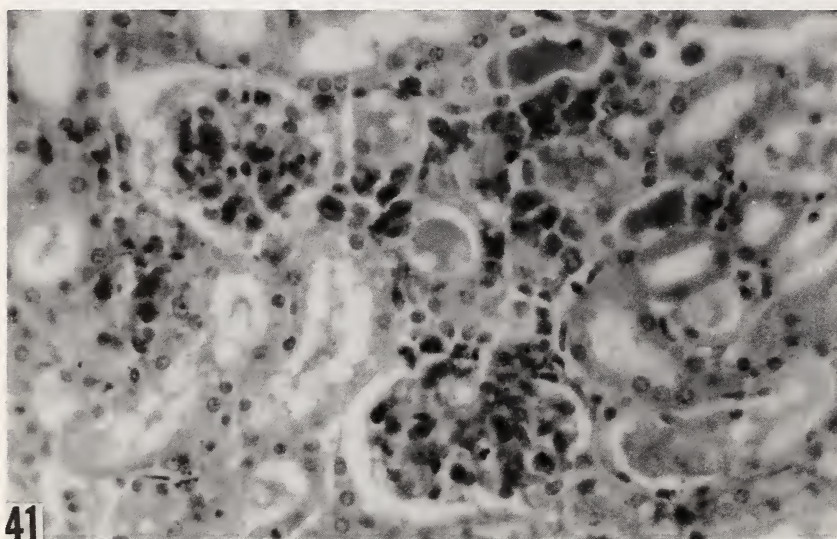
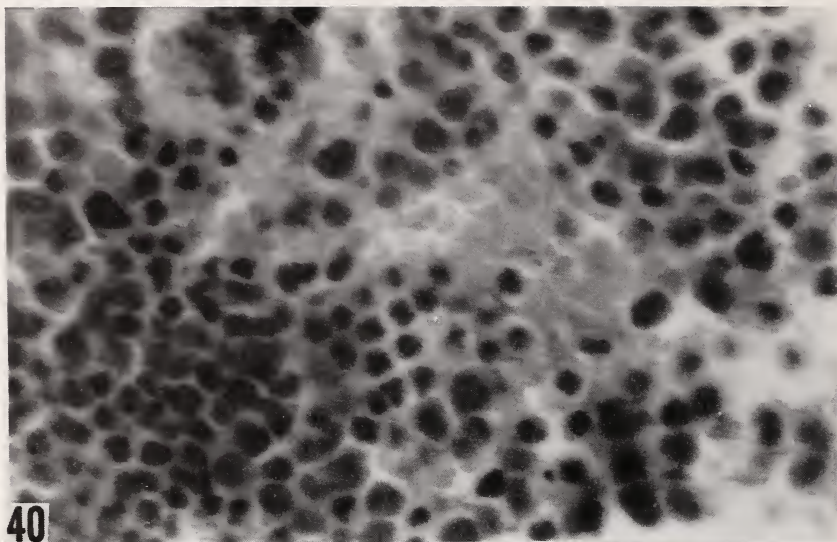


FIGURE 40.—Bone marrow with intense leukemic infiltration. Some tumor cells and immature bone marrow cells visible in the venous sinus. This could cause the myeloid reaction of the leukemic growth of SN-36.

FIGURE 41.—Kidney, 3 days after the intravenous transplantation. Many tumor cells in the glomerular tufts and other capillaries.

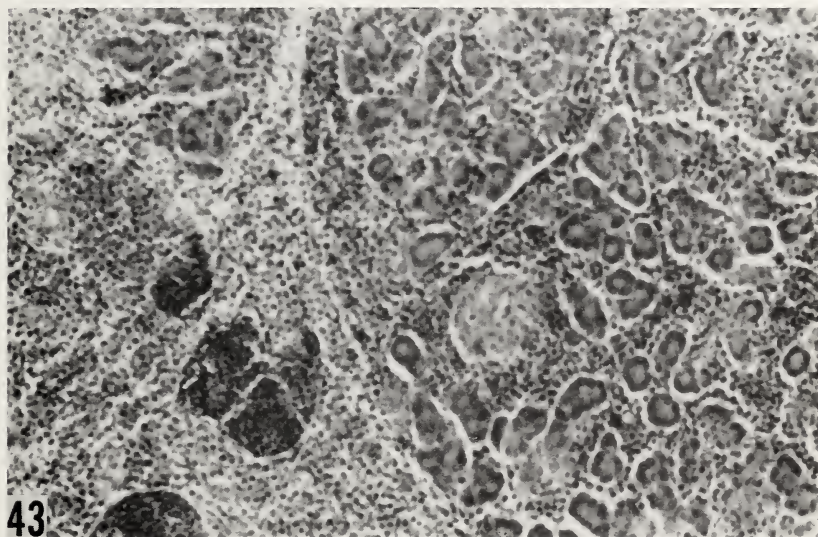
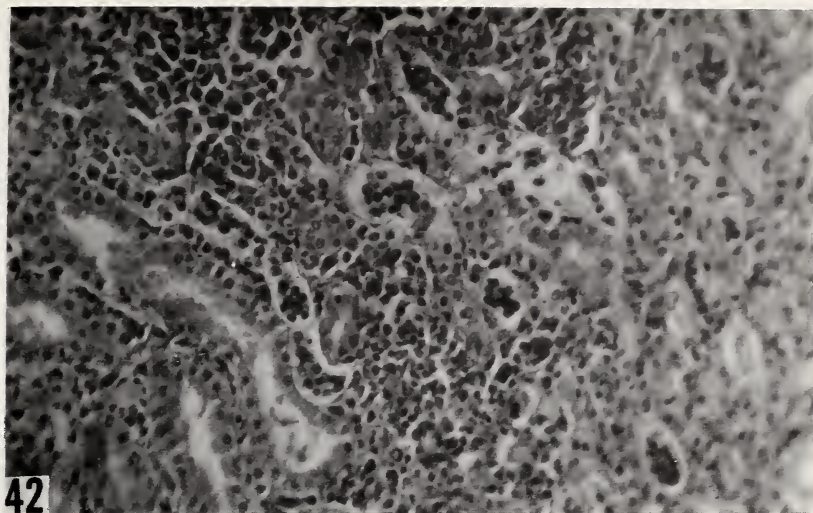


FIGURE 42.—Usual type of invasion of SN-36 into the kidney, showing diffuse infiltration in the interstitial tissue.

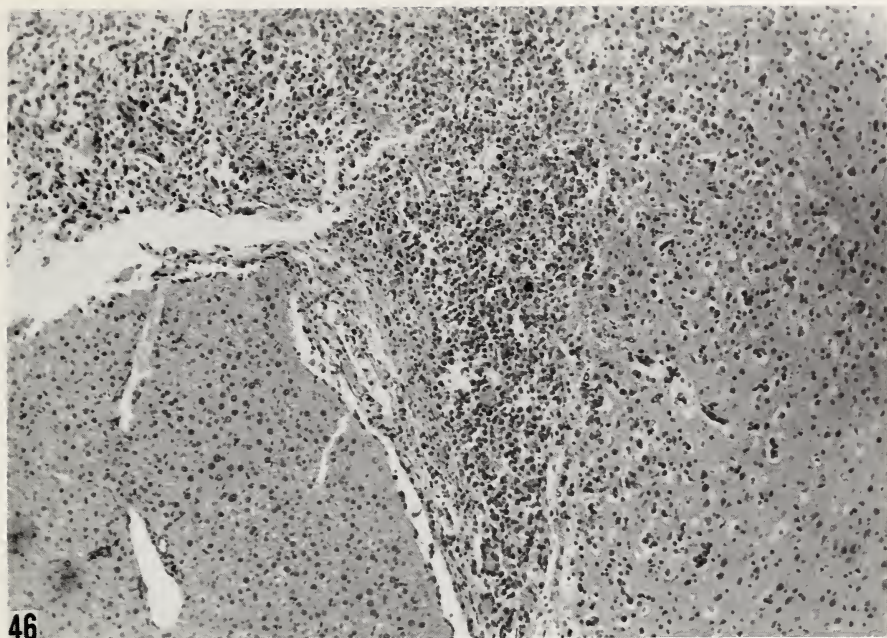
FIGURE 43.—Intense infiltration in the pancreatic tissue.



FIGURE 44.—VT-124 (*left*) transplanted intravenously and ML-230 (*right*) transplanted intraperitoneally. Both show leukemic growth similar to typical pattern of leukemic ascites tumor SN-36 in dd mice—enlargement of the liver, spleen, and mesenteric and other lymph nodes caused by leukemic invasion. Accumulation of dense tumor ascites shown in ML-230 is the only difference between the two.



FIGURE 45.—Solid-tumor formation in subcutaneous tissue after intravenous transplantation. This animal, after tumor cell inoculation, failed to develop leukemia in the organs or peripheral blood. About 10 days later, small solid tumors developed in the subcutaneous tissue. Animal died with solid-tumor growth in almost all parts of the subcutaneous tissue, still without leukemic organ infiltration. From this tumor, small pieces of tissue were transplanted into the peritoneal cavity of other dd mice in which tumor growth was typical of SN-36. Clearly, the intrinsic nature of the tumor cells was not changed.



FIGURES 46 THROUGH 49: LEUKEMIC INFILTRATION IS SHOWN IN AND AROUND GRANULAR TISSUES.

FIGURE 46.—Brain tissue present at *right* and liver tissue at *lower left*. Leukemic infiltration in granular tissue extending to the brain tissue.

FIGURE 47.—Infiltration around pieces of bone inserted in the brain.

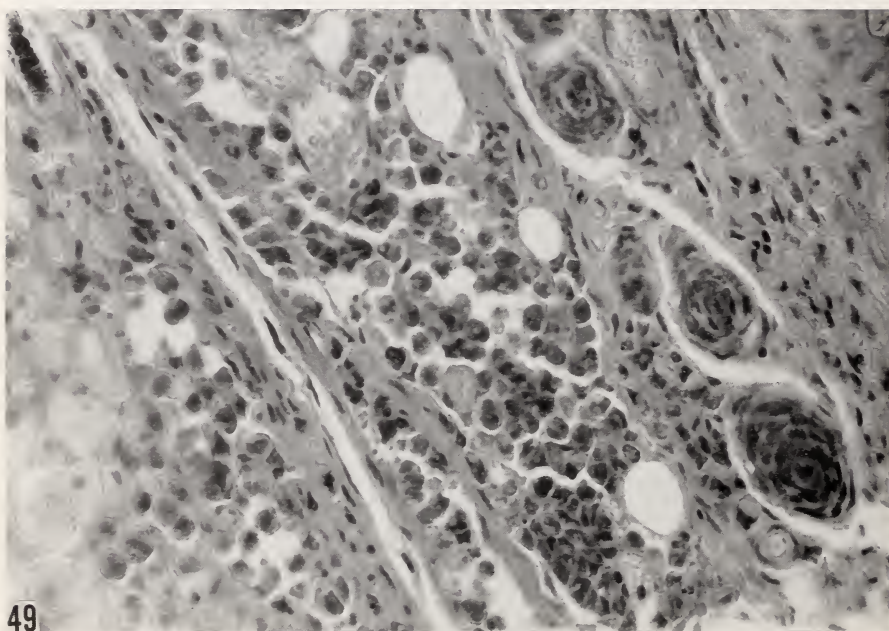
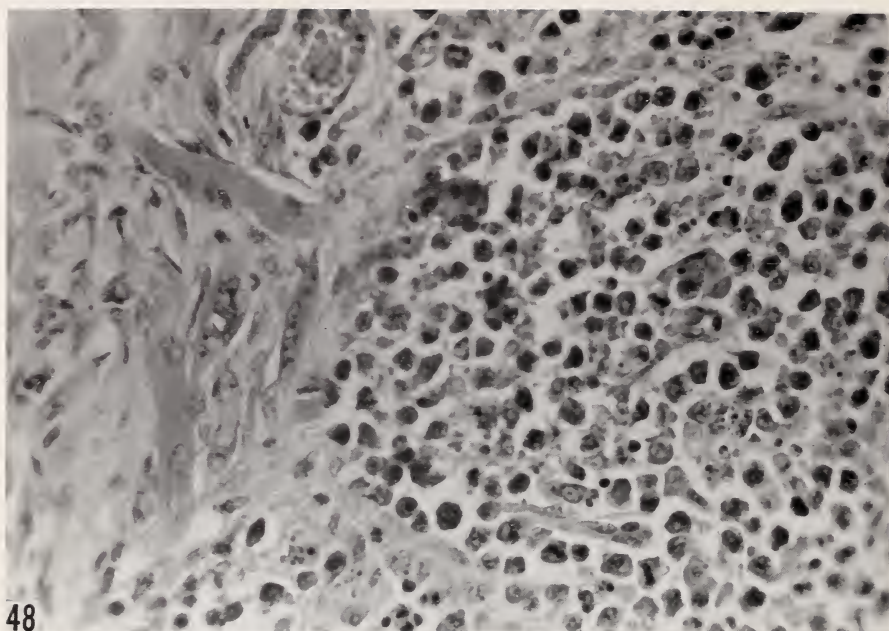


FIGURE 48.—Tumor-forming leukemic infiltration around granular tissue and degenerative muscle cells.

FIGURE 49.—Infiltration replacing granuloma formed by the injection of cinnabar into subcutaneous tissue.

## ***In Vitro Culture of Yoshida Sarcoma Cells: Methods for Determining Acquired Resistance to Drugs***<sup>1</sup>

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VARIOUS experimental tumor systems have been used to screen chemical compounds for antineoplastic activity. The criteria used for evaluating the tumor-inhibitory effect of these compounds include: 1) inhibition of tumor growth, 2) histologic changes including decrease in mitotic figures and the amount of necrosis, and 3) survival time of the host. Recent evidence has shown that tumor-inhibitory effects of certain drugs may vary depending on the host used. Thus there has been a need for an assay procedure that is capable of describing, in a quantitative manner, the relative antitumor specificity of drugs.

In 1954, Lettré and Schleich (1, 2) reported that they could culture Yoshida sarcoma cells if fibroblasts, obtained from newborn rats, were also present. We attempted to culture Yoshida sarcoma cells alone and in 1959 obtained stable growth of these cells in a comparatively simple medium (3). This resulted in the establishment (4, 5) of an experimental technique for determining the tumor-inhibitory effects of various chemical compounds. We are now studying this technique with rat ascites hepatomas. Our results suggest that the culture conditions are different for various strains of hepatomas, even with tumors induced by the same azo dye. The culture of AH 130 has been reported by Katsuta and Takaoka (6, 7). We have obtained stable growth of AH 130, AH 7974, and AH 13 (8).

With *in vitro* screening, we can observe the direct effect of the chemical on tumor cells and, also, determine the stability of the test chemical and its reaction rate. A combination of the two methods provides useful data for analyzing the activity of antitumor agents, the rapidity of the metabolic process, and the excretion of the test chemicals—especially of the transport or masked type.

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<sup>1</sup> This work was supported in part by grant CY-2799, National Cancer Institute, National Institutes of Health, Public Health Service.

<sup>2</sup> The author wishes to express his gratitude to Dr. Hidehiko Isaka for collaboration in population analysis in this work.

Actual experimentation with the cell culture and screening techniques was carried out by H. Imamura and that on the resistance problem by A. Moriwaki.

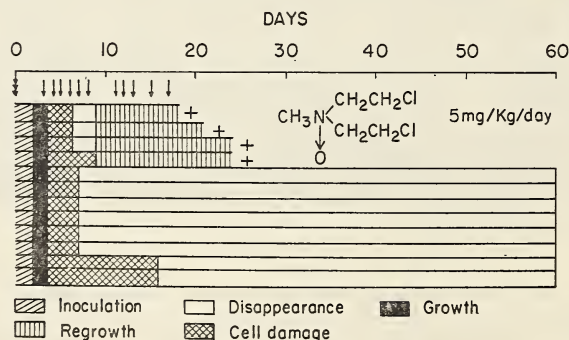
Also important in the study of cancer chemotherapy is the development of drug resistance by the tumor cells during therapy. In 1954, Hirono (9) found that tumor cells were resistant to *N*-methyl-bis(2-chloroethyl)-amine-*N*-oxide ( $\text{HN}_2\text{-O}$  or Nitromin) when it was administered continuously to rats in which Yoshida sarcoma had been serially transplanted. We confirmed this (10) and also determined that resistance to *N*-methyl-bis(2-chloroethyl)amine ( $\text{HN}_2$ ) and to nitrogen mustard derivatives could be acquired when the substances were given continuously. Even diethyl-(2-chloroethyl)amine, which alone does not show any tumor-inhibitory activity, produces resistance to  $\text{HN}_2$ . Techniques for obtaining such resistance *in vitro* were developed by use of Yoshida sarcoma cells, and this resistance could be measured by tissue culture techniques.

The data from these experiments should be useful in the search for new antitumor agents and may suggest ways to prevent the development of resistance by tumor cells or to eliminate acquired resistance. Text-figure 1 shows the development of resistance of Yoshida sarcoma cells during treatment with Nitromin in a screening procedure. In 4 of 12 rats with Yoshida sarcoma that were given 5 mg per kg per day for varying periods, there was regrowth of the tumor after the tumor cells had temporarily disappeared from the abdominal cavity (text-fig. 1). The cells from these recurrent tumors exhibited a 2.5- to 5.0-fold resistance over that of the original tumor. Since such treatment is a routine technique in screening procedures, this phenomenon is worth considering in an evaluation of antitumor activity of unknown compounds (11).

### IN VITRO CULTURE OF YOSHIDA SARCOMA CELLS

To use cultured cells as a tool for various experiments, it seemed necessary to obtain a suspended cell culture. These cultured tumor cells with no treatment will be referred to in this manuscript as the control line.

*Medium.*—It has been found that cell growth is better if chick embryo extract is not added to the medium. To a balanced salt solution with



TEXT-FIGURE 1.—Appearance of resistance of Yoshida sarcoma cells during treatment with Nitromin.

TABLE 1.—Percent composition of the medium tested\*

No.	CEE	HS	EBS	No.	CEE	HS	BS	EBS	HBS	LAH (final w/v %)
1	20	40	40	11	20		40	40		
2	10	40	50	12	10		40	50		
3	5	45	50	13	5		45	50		
4	2	48	50	14	2		48	50		
5		50	50	15			50	50		
6		40	60	16		50			50	
7		30	70	17		50†		50		
8		20	80	18		20			80	0.4
9		10	90	19		5			95	0.5
10			100							

\*Abbreviations used:

CEE: chick embryo extract

HS: horse serum

BS: bovine serum

EBS: Earle's balanced saline solution

HBS: Hanks' balanced saline solution

LAH: lactalbumin hydrolysate

†Fresh horse serum without inactivation.

horse serum and glucose, 100  $\mu$ g per ml of dihydrostreptomycin and 100 units per ml of penicillin were added to prevent bacterial growth. Various ratios of the different components tested are shown in table 1; #5 and 18 were the most satisfactory. Before use, the horse serum was inactivated by warming at 56° C for 30 minutes, but differences in the quality of the horse serum could still affect the culture growth. Therefore, a medium with 20 percent horse serum was preferable.

A stock solution of lactalbumin hydrolysate (table 1) was prepared by dissolving enzymatic hydrolysate (Nutritional Biochemicals Corp., Cleveland, Ohio) in Hanks' balanced saline at a concentration of 4 g per liter and was kept at a temperature of less than 4° C until used. Tables 2 and 3 show the actual composition of the media used.

*Number of tumor cells inoculated.*—Several drops of ascitic fluid were removed aseptically from a rat that had been inoculated 4 days previously with Yoshida sarcoma cells. The fluid was placed in a test tube containing medium and the number of cells adjusted to  $5 \times 10^4$  per ml. Good cell

TABLE 2.—Medium A

Final concentration:	Percent
Horse serum	48
Earle's balanced saline solution	50
Physiological saline solution	2
Containing:	
Penicillin G (K salt)	100 units per ml
Dihydrostreptomycin sulfate	100 $\mu$ g per ml

TABLE 3.—Medium B

Final concentration:	Percent
Horse serum	20
Hanks' balanced saline solution	78
Physiological saline solution	2
Containing:	
Penicillin G (K salt)	100 units per ml
Dihydrostreptomycin sulfate	100 $\mu$ g per ml
Lactalbumin hydrolysate	0.4%

growth can be obtained with 1 to  $5 \times 10^4$  cells per ml as the final concentration.

*Culture tubes.*—One ml of the medium was placed in each  $15 \times 90$  mm sterilized test tube and double rubber stoppers were used.

*Culture conditions.*—The tubes were placed vertically in an incubator at  $37^\circ$  C. A rotor was not used because the cells tend to aggregate in in one spot. Usually culture was continued for 120 hours without changing the medium, but for serial cultures the medium was centrifuged and changed at appropriate intervals. In one instance, culture was continued for 30 days in T-shaped flasks, with several changes of medium. Positive results were obtained when these cells were transplanted into rats.

*Cell growth.*—The growth rate of cells under these conditions is shown in table 4. Proliferation of cells slowed down markedly as the population increased and then stopped when the population reached a certain point, which indicates a close relationship between the number of cells inoculated and their proliferation over a period of time.

Table 5 shows the percentage of mitosis for cells cultured *in vitro*. Yoshida (12) found that the average mitosis of Yoshida sarcoma cells in the rat peritoneal cavity is 2.25 percent and this value agrees with that *in vitro*.

*Transplantation of cultured cells.*—The tumor cells were grown *in vitro* for 5, 10, or 14 days and then 0.5 ml of each filtrate was transplanted into the peritoneal cavity of rats. The lifespan of rats bearing this transplanted tumor and the pathologic changes observed were the same as with the control Yoshida sarcoma (table 6). There was also no difference in drug sensitivity of the cells.

TABLE 4.—Cell proliferation after incubation in medium A

Experiment	Initial No. of cells in 1 ml of medium	Number of cells in 1 ml of medium after incubation (hr)		
		24	48	72
1	$2.5 \times 10^4$	$6.3 \times 10^4$	$18.2 \times 10^4$	$25.5 \times 10^4$
2	$10.6 \times 10^4$	$27.4 \times 10^4$	$36.9 \times 10^4$	$29.7 \times 10^4$

TABLE 5.—Percent of mitoses of *in vitro*-cultured Yoshida sarcoma cells in medium A

Incubation time (hr)	Experiment No.						Average
	1	2	3	4	5	6	
24	4.4	1.5	2.8	2.2	2.3	2.1	2.6
48	1.8	2.7	2.7	1.9	2.0	1.4	2.0
72	2.7	1.9	3.7	2.2	1.8	1.3	2.3
Average	3.0	2.0	3.1	2.1	2.0	1.6	2.3

Prolonged serial cultivation of tumor cells *in vitro* often results in a change of tumor cell composition into a new cell population differing from the control cells by the effect of selection or adaptation. Therefore, the primary cell culture is better for comparison with animal experiments.

### TECHNIQUE FOR SCREENING ANTITUMOR SUBSTANCES WITH *IN VITRO* CULTURE OF YOSHIDA SARCOMA

Serial dilutions of the test substance were added to a suspended cell culture of Yoshida sarcoma and incubated for 24, 48, or 72 hours. The cells were centrifuged, stained with Giemsa, and the lowest concentration showing morphologic change was examined. This is expressed as the "minimum effective concentration" (MEC) (4). The same standard for morphologic evaluation was used as that for screening Yoshida sarcoma of the rat (13). A 48-hour culture was essential for the evaluation of most alkylating agents.

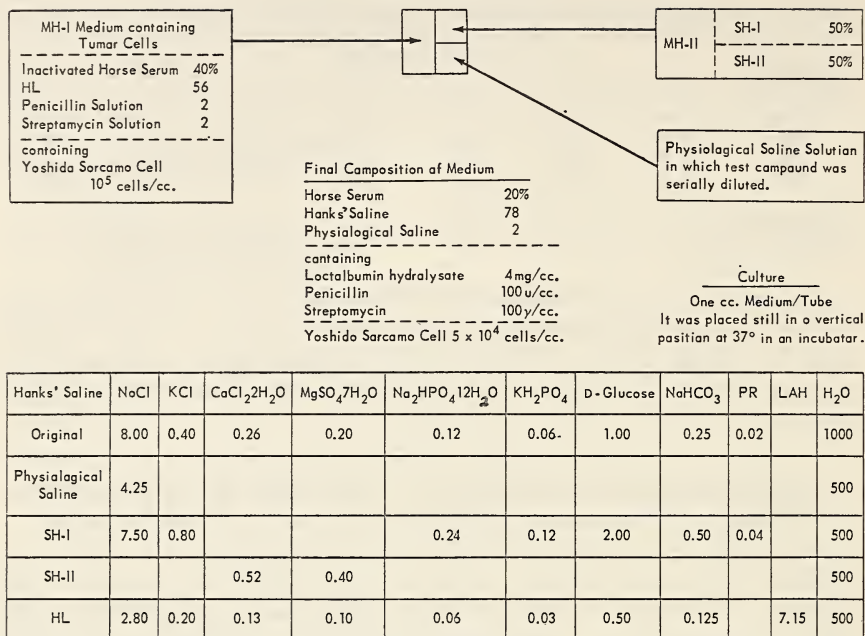
With 10 dilutions for each test agent and a sample of 24-, 48-, and 72-hour cultures for each dilution step, 30 test tubes usually were necessary. The method of assay is shown in text-figure 2.

Three test tubes were required for one step of each dilution. A fourfold dilution of the test agent was made in physiological saline and 0.75 ml of this solution was placed in a test tube. To this solution, 0.75 ml of medium II, obtained by mixing equal volumes of solutions 1 and 2 (text-fig. 2), and 1.5 ml of a  $10^5$  suspension of Yoshida sarcoma cells per ml in medium I were added at the same time. Three ml of this mixture was equally divided into 3 culture tubes to give one dilution step. A total of 30 tubes of 10-step dilutions were similarly prepared, with 3 tubes not containing any test agent for control.

The balanced salt solution (text-fig. 2) was divided into physiological saline solution and solutions 1 and 2 for the following reasons: Substances such as nitrogen mustard are stable in an acid pH range but undergo

TABLE 6.—Transplantation into rats of *in vitro*-cultured Yoshida sarcoma cells

Incubation time <i>in vitro</i> (days)	5	10	14
Transplantability	2/2	2/2	2/2



TEXT-FIGURE 2.—Assay technique of antitumor substance with *in vitro* culture (medium B) of Yoshida sarcoma.

rapid decomposition when made into a neutral or weakly alkaline solution. Consequently, addition of a buffer was avoided during the stepwise dilution and the buffer was added immediately before contact with the cells. Separation of solutions 1 and 2 was made only to increase the stability of the stock solution.

An example of the evaluation of the effect of  $\text{HN}_2$  by this method is shown in table 7. The MEC of  $\text{HN}_2$  is  $0.047 \mu\text{g}$  per ml ( $2.5 \times 10^{-4}$  mM) and this value was repeatedly reproducible.

Values of MEC for other compounds obtained by the same method are given in table 8, which indicates that the so-called antimetabolites show no MEC. This agrees with past animal experiments in which no typical morphologic changes were found in Yoshida sarcoma cells. For example, the experimental data for 6-mercaptopurine elicited cytolysis only in a high concentration (table 9), and it was necessary to evaluate the carcinolytic effect by the way the agent affected the growth of the culture cells, as will be described.

The value of MEC tends to differ according to the medium used so that the comparison of the effect of various substances must be made with the same medium. For example, the effect of  $\text{HN}_2$  in medium B containing lactalbumin hydrollysate gave an MEC value of  $1 \times 10^{-4}$  mM.

In this culture, addition of an antitumor substance to the medium resulted in the lowering of cell proliferation. The proliferation rate on the addition of  $\text{HN}_2$  is shown on the left in text-figure 3. Growth of the cells at

TABLE 7.—Determination of MEC\* (medium A)

Compound	Incu- bation (hr)	No. $\mu\text{g}/\text{ml}$ mm	1	2	3	4	5	6	7	8	9	10	Control
			19	9.5	4.75	1.9	0.95	0.475	0.19	0.095	0.0475	0.019	0
			$1 \times 10^{-1}$	$5 \times 10^{-2}$	$2.5 \times 10^{-2}$	$1 \times 10^{-2}$	$5 \times 10^{-3}$	$2.5 \times 10^{-3}$	$1 \times 10^{-3}$	$5 \times 10^{-4}$	$2.5 \times 10^{-4}$	$1 \times 10^{-4}$	
			Cytolysis							Normal mitoses			
$\text{CH}_3\text{N} \begin{array}{l} \diagup \text{CH}_2\text{CH}_2\text{Cl} \\ \diagdown \text{CH}_2\text{CH}_2\text{Cl} \\ \cdot \text{HCl} \end{array}$ $(\text{HN}_2)$ mol. wt. 189.5	24												
	48		Cytolysis							Abnormal mitoses		Normal mitoses	
	72		Cytolysis							Degeneration		Normal mitoses	

\* MEC:  $2.5 \times 10^{-4}$  mM (0.0475  $\mu\text{g}$  per ml).

TABLE 8.—Minimum effective concentration (MEC) of the known antitumor agents (medium A)

Compound No.	Compound	Cytological effect	MEC	
			mm	μg per ml
24	Nitroimin	+	$2.5 \times 10^{-4}$	0.05
556	HN <sub>2</sub> -picrate	+	$2.5 \times 10^{-4}$	0.096
191	TEM	+	$5 \times 10^{-5}$	0.01
305	ThioTEPA	+	$5 \times 10^{-4}$	0.095
263	6-Mercaptopurine	—		
242	8-Azaguanine	—		
239	Glucosamine	—		
555	5-Fluorouracil	—		
479	Carcinophillin	+		2.5 (units/ml)
584	Mitomycin C	+		0.01

MEC, *i.e.*, at  $1 \times 10^{-4}$  mm concentration of HN<sub>2</sub>, is shown by line number 7. That the lowering of proliferation rate is proportional to the concentration of the agent, within a certain limit, is shown on the right in text-figure 3. Consequently, the concentration at which the cell growth fell to 50 percent of the controls after 48 hours was constant, and this value is called the 50 percent inhibition concentration (IC<sub>50</sub>) (5). The IC<sub>50</sub> of HN<sub>2</sub> was  $1 \times 10^{-5}$ , approximately one tenth the concentration of MEC.

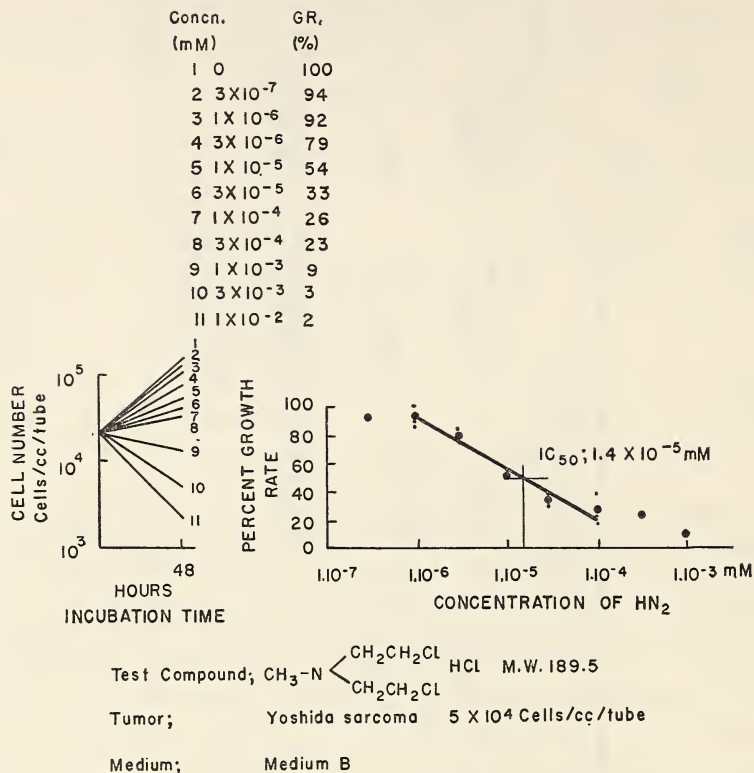
The composition of the medium used is also given in text-figure 3. Usually the initial concentration of the cells was maintained at about  $2.5 \times 10^4$  cells per ml, but when data for long-term incubation are required the number of cells in the inoculum should be decreased.

After incubation, the culture medium was centrifuged and 2 ml of 0.1 M citric acid solution, in which crystal violet was dissolved in 0.05 percent ratio, was added, and the mixture was shaken at 37° for 30 minutes. A part of this solution was used in a Bürker hemocytometer to count the number of cells. The rate of control growth was taken as 100, and the growth rate of each dilution step calculated, from which the IC<sub>50</sub> was calculated. Within a certain dilution step, the dose-response curve was linear, *e.g.*, a linearity was established between  $1 \times 10^{-6}$  and  $1 \times 10^{-4}$  mm in HN<sub>2</sub>. When test agents are sparingly soluble in water, they can be dissolved in ethanol and diluted with the medium, but it is necessary to limit the final concentration of ethanol to less than 0.3 percent. The IC<sub>50</sub> of some antimetabolites against Yoshida sarcoma was measured and the results are presented in text-figures 4 and 5.

Evaluation of the effect of various antitumor substances by screening with transplanted animal tumor and the present *in vitro* tests do not necessarily agree. When antineoplastic compounds are administered systemically to tumor-bearing animals, the metabolic pathway and metabolites formed may differ depending on the laboratory animal used. Direct contact of the agent with the tumor cell is probably limited. An analysis of this has been made by Ishidate *et al.* (14). In general, the

TABLE 9.—Morphologic changes of Yoshida sarcoma cells *in vitro* by 6-mercaptopurine (medium A)

Tube No.	Concentration		Incubation time (hr)				
	mm	$\mu\text{g/ml}$	24	48	72	96	120
1	$1 \times 10^{-1}$	15	No observable change	Cytolysis	Cytolysis	Cytolysis	Cytolysis
2	$5 \times 10^{-2}$	7.5	"	Huge cell	"	"	"
3	$2.5 \times 10^{-2}$	3.75	"	"	Huge cell	"	"
4	$1 \times 10^{-2}$	1.5	"	No observable change	"	Huge cell	Huge cell
5	$5 \times 10^{-3}$	0.75	"	"	"	"	"
6	$2.5 \times 10^{-3}$	0.375	"	"	"	"	"
7	$1 \times 10^{-3}$	0.15	"	"	No observable change	No observable change	No observable change
8	$5 \times 10^{-4}$	0.075	"	"	"	"	"
9	$2.5 \times 10^{-4}$	0.0375	"	"	"	"	"
10	$1 \times 10^{-4}$	0.015	"	"	"	"	"
Control	0	0	"	"	"	"	"



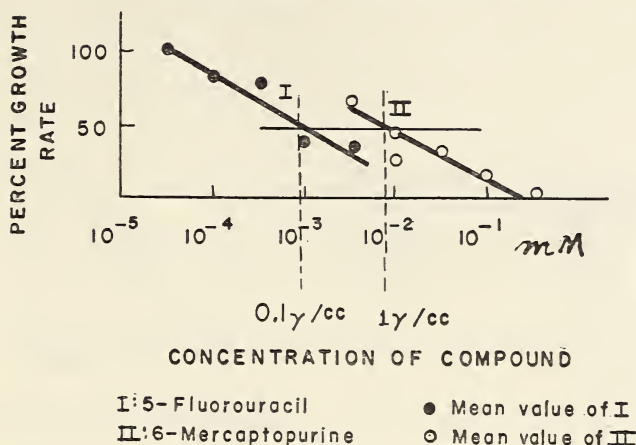
TEXT-FIGURE 3.—Estimation of 50 percent inhibition concentration (IC<sub>50</sub>) by cell counting.

present *in vitro* test is more sensitive than animal experiments for observing the direct action of a chemical agent on tumor cells, and it is one of the best methods for preliminary screening of unknown substances.

## STUDIES ON THE ACTIVATION CONDITIONS FOR MASKED DERIVATIVES

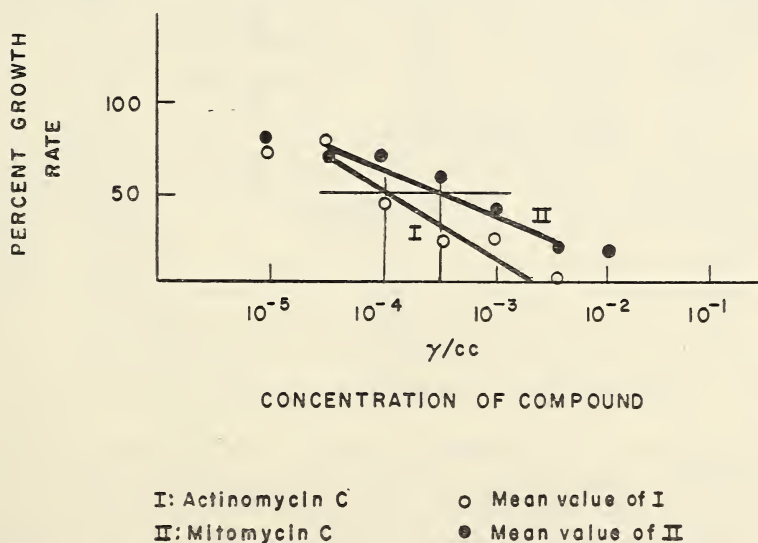
Efforts to increase the selective action of antitumor agents by synthesizing them from masked derivatives or derivatives with latent activity have been made, not only in Japan but also in England and Germany. Unless we know that the compound, when introduced into the animal body, will be activated by the tumor the significance of latent activity will usually be lost. It is better if the agent is rather rapidly activated by the tumor tissue alone and not so actively by normal tissue.

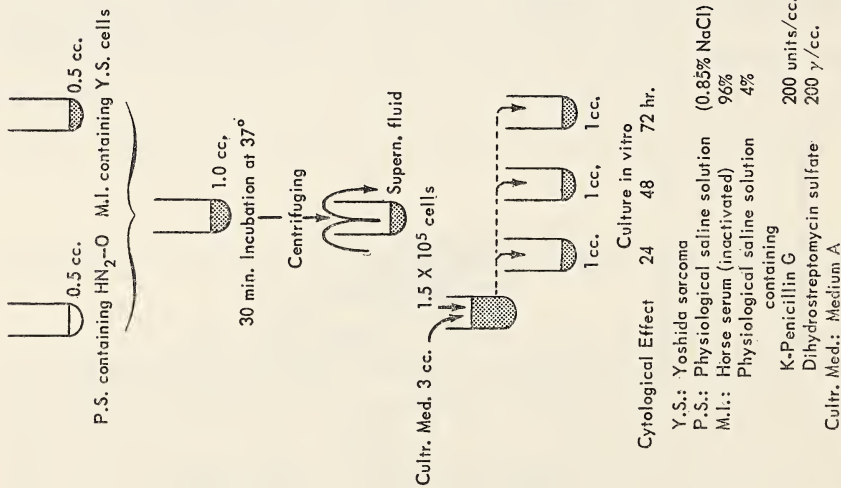
It would be interesting to expose the masked derivatives showing good therapeutic effect, when given to animals with Yoshida sarcoma, to Yoshida sarcoma cells *in vitro* and to measure the progress of their activation so their effectiveness could be compared with that of animal ex-

TEXT-FIGURE 4.—Determination of IC<sub>50</sub> of 5-fluorouracil and 6-mercaptopurine.

periments. Imamura (15) clarified the conditions for activation of the masked derivative Nitromin by using several series of stepwise dilutions of Nitromin in culture medium and incubating the tubes with different cell populations of Yoshida sarcoma. MEC was microscopically measured after 48 hours with the cells from each combination. The methods and results are shown in text-figure 6.

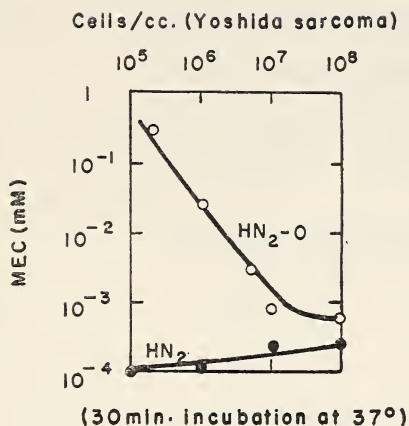
Nitromin showed little effect in a small cell population of Yoshida sarcoma but, in populations sufficient to activate the agent, was observed in up to  $5 \times 10^{-4}$  mM (text-fig. 6). Since the active substance of Nitromin is  $\text{HN}_2$  and the MEC of  $\text{HN}_2$  is  $2.5 \times 10^{-4}$ , the agent is completely activated at a cell population of  $10^8$  cells per ml. In a similar manner, the

TEXT-FIGURE 5.—Determination of IC<sub>50</sub> of actinomycin C and mitomycin C.



Cells/cc. final	mm final	Yoshida Sarcoma Cells				
		$2 \times 10^5$	$1 \times 10^6$	$5 \times 10^6$	$1 \times 10^7$	$1 \times 10^8$
1		+				
$5 \times 10^{-1}$		+				
$2.5 \times 10^{-1}$		+				
$1 \times 10^{-1}$		-	+	+	+	+
$5 \times 10^{-2}$		-	+	+	+	+
$2.5 \times 10^{-2}$		-	+	+	+	+
$1 \times 10^{-2}$		-	-	+	+	+
$5 \times 10^{-3}$		-	-	+	+	+
$2.5 \times 10^{-3}$		-	-	+	+	+
$1 \times 10^{-3}$		-	-	-	+	+
$5 \times 10^{-4}$		-	-	-	-	+
$2.5 \times 10^{-4}$		-	-	-	-	-
$1 \times 10^{-4}$		-	-	-	-	-

TEXT-FIGURE 6.—Shifting of minimum effective concentration of Nitromin with increase of tumor cell population.



TEXT-FIGURE 7.—Shifting of the minimum effective concentration with increase of tumor cell number with  $\text{HN}_2$  and Nitromin.

effect of  $\text{HN}_2$  which is not a masked derivative, on the cell population was observed and the result is shown in text-figure 7. There was almost no effect on cell population; the value of MEC tends to increase with the increasing cell population.

It would be possible by this method to make qualitative and quantitative determinations of the substance activated *in vitro*. Nitromin is activated not only by Yoshida sarcoma but also by tissue slices of rat kidneys, liver, spleen, and heart, and this reaction was not specific for tumor tissue. Interestingly, the activation efficiency of Nitromin-resistant and control Yoshida sarcoma is the same as for Nitromin. This indicates that the principle of resistance against Nitromin is not only a phenomenon seen through the activation mechanisms but also one of cell permeability, which differs slightly between the resistant and control tumor cells, if one assumes that this activation takes place inside the cell membrane.

*N,N*-bis(2-chloroethyl)*N'*,*O*-propylene phosphoric acid ester diamide monohydrate (Endoxan) is one of the most promising masked derivatives of the nitrogen mustard series now available. This substance gave excellent therapeutic results in rats bearing Yoshida sarcoma, but *in vitro* results were negative. No activation was observed even when the cell population was increased to  $10^8$  cells per ml, but the agent was easily activated by liver slices to expose the active moiety. Activation mechanism of Endoxan showed no useful conditions for tumor selectivity and yet its therapeutic effect is much better than the original nitrogen mustards.

Also, the possible active moiety of Endoxan is a secondary amine, *N*-bis(2-chloroethyl)amine, whose antitumor effect against Yoshida sarcoma is very weak (table 10). Many questions remain, but this fact suggests that, in masked derivatives, the agent as a whole may prove very effective even if its active moiety alone is not expected to have much

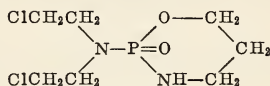
TABLE 10.—Effect of Endoxan on Yoshida sarcoma

	LD50*	MTD†	MED‡
Endoxan	175	100	1.0
N-bis(2-chloroethyl) amine	300	100	50

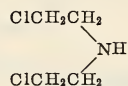
\*LD50: mg/kg intraperitoneal

†MTD: Maximum tolerated dose mg/kg intraperitoneal

‡MED: Minimum effective dose mg/kg intraperitoneal



Endoxan



N-bis(2-chloroethyl)amine

antitumor effect. Thus it is important to synthesize this type of antitumor agent.

#### IN VITRO INDUCTION OF ACQUIRED RESISTANCE OF YOSHIDA SARCOMA TO VARIOUS AGENTS

The development of resistance of Yoshida sarcoma cells to various agents *in vitro* was performed as follows (16): One volume of ascitic fluid from a Yoshida sarcoma rat was mixed with an equal volume of physiological saline solution in which was dissolved the desired concentration of  $\text{HN}_2\text{-HCl}$ . The final cell population was  $10^8$  cells per ml. The concentration of the drug used was either one half or several times the MEC. This mixture was maintained at  $37^\circ$  for 30 minutes and then centrifuged at 2000 rpm for 20 seconds; the residue was washed with physiological saline solution. The cells were transplanted into the peritoneal cavity of a healthy rat. After growth, the cell population had a resistance several times that of the parent population. Results of 15 experiments with different concentrations of tumor cells after a single contact with  $\text{HN}_2$  are shown in table 11.

TABLE 11.—Resistance induced in Yoshida sarcoma after a single exposure to  $\text{HN}_2$ 

Concentration of $\text{HN}_2$ (final mM)	Resistance index MEC- $\text{HN}_2$	Number of cases
	1	1
$2.5 \times 10^{-4}$	2.5	6
	5	3
$5 \times 10^{-4}$	2.5	3
	5	1
$1 \times 10^{-3}$	2.5	1

TABLE 12.—Development of resistance of the original Yoshida sarcoma after repeated exposure *in vitro* to HN<sub>2</sub>

Repetition of treatment	Experiment 1		Experiment 2	
	Concentration of HN <sub>2</sub> (final mM)	Resistance index MEC-HN <sub>2</sub>	Concentration of HN <sub>2</sub> (final mM)	Resistance index MEC-HN <sub>2</sub>
Untreated		1		1
1	$5 \times 10^{-4}$	5	$2.5 \times 10^{-4}$	5
2	$2 \times 10^{-3}$	5	$2.5 \times 10^{-4}$	5
3	$2.5 \times 10^{-3}$	10	$1 \times 10^{-3}$	10
4	$2.5 \times 10^{-3}$	25	$5 \times 10^{-3}$	100
5	$5 \times 10^{-3}$	100	$1 \times 10^{-2}$	100
6	$1 \times 10^{-2}$	100	$5 \times 10^{-2}$	500
7	$2.5 \times 10^{-2}$	250		
8	$5 \times 10^{-2}$	500		
9	$1 \times 10^{-1}$	500		
10	$1 \times 10^{-1}$	1,000		
11	$1 \times 10^{-1}$	1,000		
12	$2.5 \times 10^{-1}$	1,000		
13	$5 \times 10^{-1}$	5,000		
14	1	10,000		
15	1	10,000		
16	2.5	10,000		
17	2.5	10,000		
18	2.5	10,000		
19	2.5	10,000		
20	2.5	25,000		
21	2.5	25,000		
22	2.5	25,000		

Table 12 shows the results of repeated *in vitro* contact of the resistant tumor to HN<sub>2</sub>, in which a 25,000-fold increase in resistance was effected by 22 exposures.

Exposure of a tumor, having a tenfold resistance to HN<sub>2</sub>, to ethyl-bis-(2-chloroethyl)amine (Et-HN<sub>2</sub>) twice *in vitro* resulted in an increase of the original resistance to Et-HN<sub>2</sub> but no change in resistance to HN<sub>2</sub> (table 13). Strain RA had a tenfold resistance to HN<sub>2</sub> and a fourfold resistance to Et-HN<sub>2</sub>, while RA<sub>EC-2</sub> after two exposures to  $2.5 \times 10^{-3}$  mM of Et-HN<sub>2</sub> had a 20-fold resistance to Et-HN<sub>2</sub>, but the resistance to HN<sub>2</sub> remained tenfold. These results indicate that there was some degree of specificity in resistance of cells exposed to chemical agents *in vitro*.

TABLE 13.—Specificity of resistance-inducing agent

Tumor	Resistance index	
	MEC-HN <sub>2</sub>	MEC-Et-HN <sub>2</sub>
RA	10	4
RA <sub>EC-2</sub>	10	20
RA <sub>C-7</sub>	1000	40
Yoshida sarcoma	1	1
	(MEC 1 $\times 10^{-4}$ mM)	(MEC 2.5 $\times 10^{-3}$ mM)

TABLE 14.—Development of resistance after exposure to mitomycin C

Repetition of treatment	Concentration of mitomycin C ( $\mu\text{g/ml}$ )	Subline	Resistance index	
			MEC-mitomycin C	MEC- $\text{HN}_2$
		Original Yoshida sarcoma	1	1
1	2.5	YS <sub>MC-1</sub>	1	1
2	5	YS <sub>MC-2</sub>	1	1
3	5	YS <sub>MC-3</sub>	1	1
4	20	YS <sub>MC-4</sub>	4	5
5	40	YS <sub>MC-5</sub>	4	5

Induction of resistance by exposure of Yoshida sarcoma to mitomycin C *in vitro* was more difficult than that with  $\text{HN}_2$ , but a fourfold resistance was induced (table 14). An abnormally high concentration of mitomycin C was required. This indicates that the action of mitomycin is so slow that a high concentration is necessary to produce an effect on the cells during the 30-minute exposure time.

Control Yoshida sarcoma acquired a 25,000-fold resistance to  $\text{HN}_2$  after being exposed more than 20 times.

We cannot elucidate the mechanism of this induced resistance but will discuss it later.

Induction of resistance *in vitro* by methods other than exposure to the agent might also be of interest. The resistant line of Yoshida sarcoma RA<sub>C-20</sub> or rat ascites hepatoma, AH 7974, which are strongly resistant to  $\text{HN}_2$ , were cultivated *in vitro* for 48 hours in the medium reported by Ishidate *et al.* (3). The cell-free filtrate was then used for a second 48-hour culture of the normal Yoshida sarcoma cells. After incubation, the cultured cells were transplanted into healthy rats and the newly grown cell population was assayed for its resistance index to  $\text{HN}_2$ . The filtrate of the primary culture of the original Yoshida sarcoma was also used as the control. The data in table 15 were analyzed, and there was no significant difference between the mean value of IC<sub>50</sub> obtained in 3 different experiments. The second experiment was done to examine the

TABLE 15.—Transforming activity of culture broth obtained by tissue culture of resistant tumor

IC <sub>50</sub> of $\text{HN}_2$ against Yoshida sarcoma				
	Basal medium	Broth of RA <sub>C-20</sub>	Broth of AH 7974	Broth of Yoshida sarcoma
	1.2	2.0	1.9	1.4
	1.6	1.4	2.6	1.4
	2.0	1.0	1.7	1.0
	1.0		1.9	
	1.1			
	1.2			
	1.4			
Average	1.4	1.5	2.0	1.3

TABLE 16.—Transforming activity of DNA obtained from resistant tumors

Source of DNA	Incubation time (hr)	Cell population (cells/ml)	Final concentration of DNA (approx %)	Rat No., transplan- tation	Resistance index MEC-HN <sub>2</sub>
RAC-2 (Resistance index: 100)	1	$2 \times 10^5$	0.5	T-1463	1
	4			T-1465	1
	24	$5 \times 10^4$		T-1646	1
	48			T-1467	1
	72			T-1468	1
RAC-5 (Resistance index: 400)	1	$2 \times 10^5$	0.5	T-1531	1
	2			T-1532	1
	4	$5 \times 10^4$		T-1533	1
	24			T-1534	1
	48			T-1537	1
	72			T-1540	1

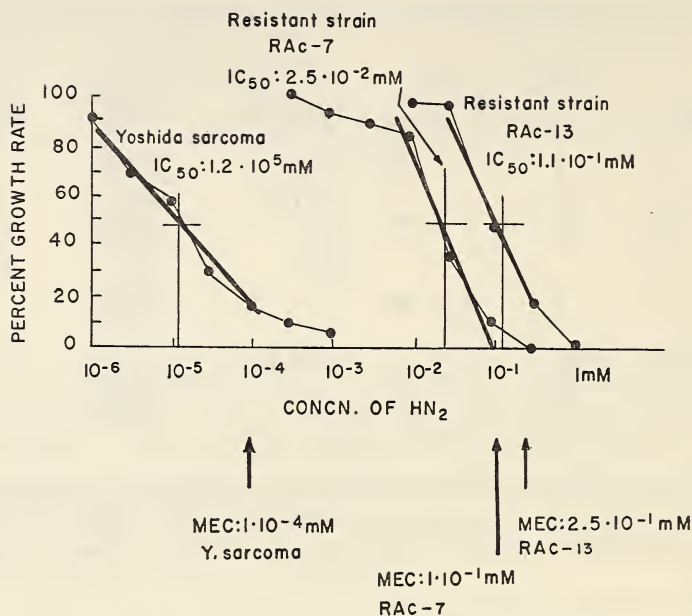
transforming activity *in vitro* of DNA extracted from the resistant lines of Yoshida sarcoma. Experimental conditions and results are given in table 16, but none of the experiments showed transformation of resistance.

## MEASUREMENT OF RESISTANCE

Measurement of resistance of a tumor has previously been made by transplantation of the tumor into an animal and administration of a definite quantity of a drug. The rate of tumor growth, morphologic changes, and host survival time were compared with the findings in the non-resistant tumor-bearing animals. This method though does not give quantitative data.

With Yoshida sarcoma, however, resistance has been demonstrated quantitatively through *in vitro* culture of the cells. It is possible to obtain MEC or IC<sub>50</sub> of the test chemical, and to calculate the ratio of these values with those obtained with a nonresistant strain (17). For example, if the MEC of an HN<sub>2</sub>-resistant strain is 100 times that of the nonresistant strain, the resistance ratio is expressed as 100. IC<sub>50</sub> can be similarly compared; results from experiments on a few resistant strains are shown in text-figure 8.

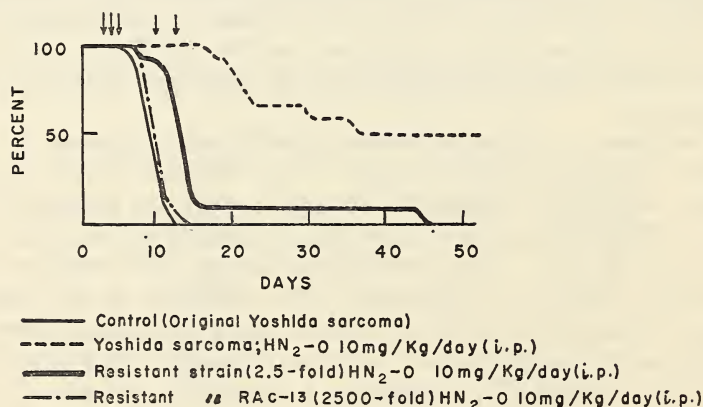
The resistance of nitrogen mustard derivatives expressed by MEC and IC<sub>50</sub> ratios compares favorably. The MEC is usually found in the lower part of the response curve, and it would be natural that the MEC and IC<sub>50</sub> do not necessarily agree according to the value of the tangent (text-fig. 18). This straight line changes also with the degree of uniformity of the cell population. The difference in the degree of resistance expressed by these two values might give some information regarding the cell composition of the resistant tumor. To find the significance of such values of resistance as measured by the use of *in vitro* culture, some experiments were carried out on the therapy of tumor-bearing animals (text-fig. 9).



TEXT-FIGURE 8.—Determination of resistance index.

Nitromin was administered under conditions in which it would show sufficient therapeutic effect against the Yoshida sarcoma with a 2.5-fold resistance to  $HN_2$ . This will show the degree of resistance acquired by the 100-fold and 1,000-fold resistant strains mentioned. Such a high degree of resistance can only be measured by an *in vitro* culture method because the necessary concentration of the drug is far above the lethal dose in these animals.

Evaluation of the resistance of a tumor, when a tumor cell is proliferating in the host, by the effect of a chemical agent on it is an effective method,



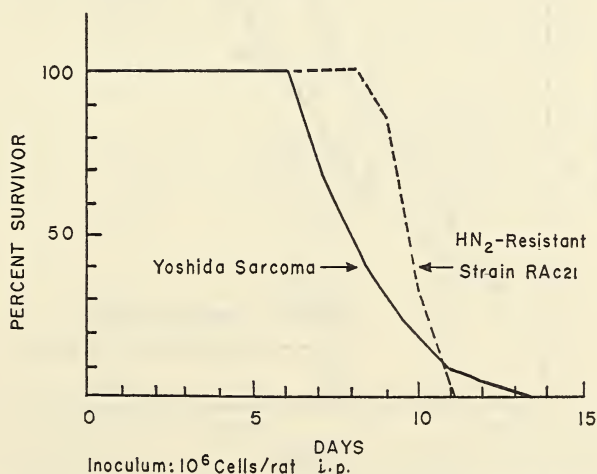
TEXT-FIGURE 9.—Percentage survival with a normal and resistant strain of Yoshida sarcoma treated with Nitromin.

but it has recently been shown that the appearance of a drug effect is dependent on the host-tumor relationship. Consequently, the *in vitro* measurement of resistance is an excellent method because it can standardize resistance as a property of a cell. Application of  $\text{HN}_2$  to *in vitro* cultures of Yoshida sarcoma cells in far higher concentration than its MEC, in  $10^{-2}$  mM, results in complete cytolysis after 24 to 48 hours. This is called a cytolytic concentration (CyC). Resistance of a cell to  $\text{HN}_2$  results naturally in the increase of CyC with increase of MEC. For example, cytolysis occurs first at a concentration of 2.5 mM in 10,000-fold resistant cells. Although it is difficult to make exact measurement of CyC, this is an interesting fact.

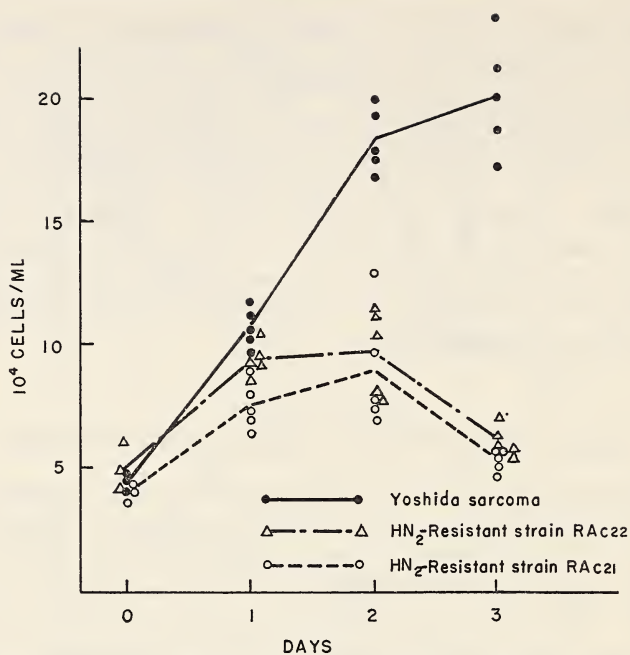
### CHARACTERISTICS OF RESISTANT TUMORS

The lifespan of rats inoculated with a resistant line of Yoshida sarcoma,  $\text{RA}_{\text{C-21}}$ , was slightly longer than that of the original Yoshida sarcoma (text-fig. 10). Because of this finding, the growth rate *in vitro* of resistant lines  $\text{RA}_{\text{C-21}}$  and  $\text{RA}_{\text{C-22}}$  was examined with the medium previously mentioned. With the same number of cells as inoculum, the growth rates with these two resistant strains and the control strain were very different (text-fig. 11). However, the result of a similar experiment with the other resistant strain  $\text{YS}_{\text{C-15}}$  (resistance index: 10,000) was reversed. The growth rates of both resistant and nonresistant lines were almost the same (text-fig. 12). According to these results, growth rate seemed to be an attribute of each line and did not show correlation with its drug resistance.

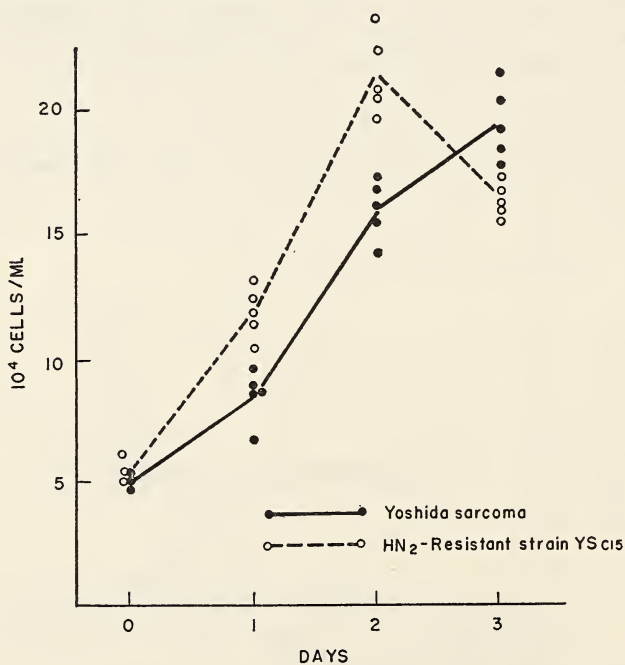
The resistant tumors obtained by the foregoing method were transplanted serially, via the peritoneal route, into rats and changes in their



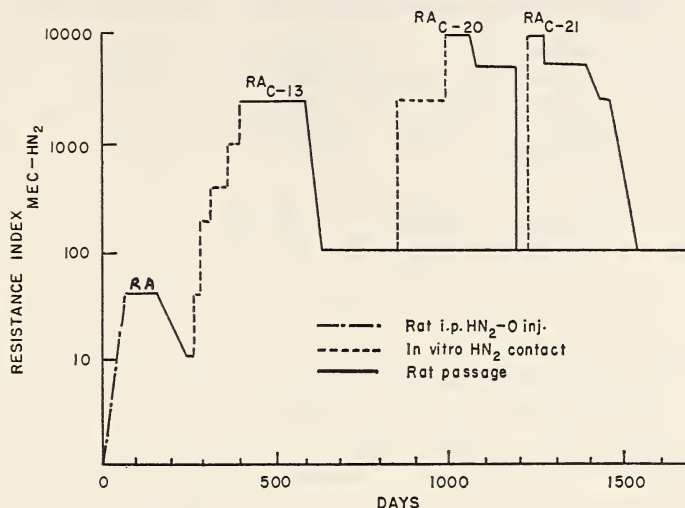
TEXT-FIGURE 10.—Percentage survival of control and resistant lines of Yoshida sarcoma.



TEXT-FIGURE 11.—Growth rate *in vitro* of control and resistant lines of Yoshida sarcoma.



TEXT-FIGURE 12.—Growth rate *in vitro* of control and resistant lines of Yoshida sarcoma.



TEXT-FIGURE 13.—Change in resistance index during passage. *Dotted line* shows induction of resistance after exposure to  $\text{HN}_2$ ; *solid line* shows period during which cells were serially transplanted without treatment.

resistance were observed (text-fig. 13). The acquired resistance was maintained for some time during serial passage, but decreased after a few months (table 17).

This spontaneous fall of resistance during passage was also observed even with single cell clones obtained from resistant strains of this tumor (table 18), which might indicate that the shift of composition of the original mosaic formation of tumor cells was not the only cause of decrease of the index.

Table 19 shows an interesting comparison between two resistance indices obtained from the determinations of MEC and  $\text{IC}_{50}$  ( $\text{RI}_{\text{MEC-HN}_2}$ ,

TABLE 17.—Period of continuation of high resistance

Resistant lines	Resistance index MEC- $\text{HN}_2$	Period of continuation of resistance (months)
RAC-13	2500	6.5
	100	8
RAC-20	10,000	2
	5000	4
	100	18
YSC-5	100	2
	25	4
	10	5
YSC-15	10,000	2
	5000	1
	2500	3

TABLE 18.—Decrease in resistance index of single cell clones

Control population	Clone	Change of resistance index	Period (months)
RA <sub>C-13</sub> (resistance index: 2500)	G II	2500→100	4. 5
	G III	2500→100	
RA <sub>C-13</sub> (resistance index: 100)	SG 681	100→50	2. 5
	SG 677	50→25	
	SG 726	250→50	
	SG 694	100→25	

RI<sub>IC50-HN<sub>2</sub></sub>) of a resistant line. Resistant line RA<sub>C-20</sub> began to lose its resistance on December 9, 1962, when the resistance indices determined by two different methods were quite dissimilar, but 9 days later both indices were almost the same. The status of the tumor on December 9, 1962, indicated that it was in a transient phase, in which the cells of low resistance were appearing in its original population.

When the lowest resistance index became comparatively stable, there was no change in resistance for over a year. As shown in table 19, exposure of the cells to the chemical agent *in vitro*, soon after this resistance index was reached, resulted in easy reversion of the resistance to the original level or higher, but if it was left for long at the low resistance level, recovery of resistance was not so feasible and repeated exposure was needed (table 20). It would be difficult to postulate the significance of this phenomenon, but as will be shown later such an experimental result is of interest.

Isaka transplanted one cell from a Yoshida sarcoma, and from resistant tumors of various degrees (text-fig. 14), into the peritoneal cavity of a healthy rat and obtained a clone whose resistance was measured by the foregoing *in vitro* method. Table 21 shows the result of population analysis of the control line of Yoshida sarcoma. Sixteen clones were established and measurement of their resistance showed 13 clones with a resistance of 1 and 3 clones with a 2.5-fold resistance.

Population analyses of resistant line RA<sub>C-13</sub> of Yoshida sarcoma with a 2,500-fold resistance and clone RA<sub>C-13</sub>, established by its serial passage, with a decrease of resistance to 100-fold are shown in text-figure 14. The figures on the ordinate are the resistance indices; the population analyses were carried out at the point of the arrows.

TABLE 19.—Decrease in resistance index of a resistant line

RA <sub>C-20</sub>	Resistance index	
	MEC-HN <sub>2</sub>	IC50-HN <sub>2</sub>
Experiment 1	5,000	10,000
Experiment 2 (12/9/62)	5,000	400
Experiment 3 (12/18/62)	100	200

TABLE 20.—Recovery of resistance after exposure to chemicals

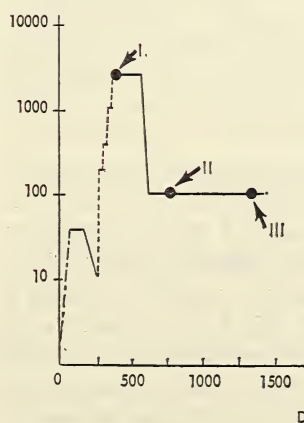
	Period of low resis- tance (days)	Concentration of a single ex- posure (mM)	Resistance index MEC-HN <sub>2</sub> after treatment
RA <sub>C-13</sub>	233	$1 \times 10^{-2}$	2,500
Resistance index: 2,500 ↓	720	$1 \times 10^{-2}$	100
Resistance index: 100	770	$1 \times 10^{-2}$	100
RA <sub>C-20</sub>	30	$5 \times 10^{-1}$	10,000
Resistance index: 10,000 ↓	120	$5 \times 10^{-1}$	5,000
Resistance index: 100	210	$5 \times 10^{-1}$	100

Experiment III in text-figure 14 indicated that the composition of cells of RA<sub>C-13</sub> changed during passage of approximately 1½ years, without change in resistance index of a whole population. The shift in composition of the cell population during passage in rats also seemed possible from the following results.

Exposure of RA<sub>C-13</sub>, with 100-fold resistance, to NH<sub>2</sub> *in vitro* resulted in clone RA<sub>C-14</sub> with a 1,000-fold increase in resistance. Text-figure 15 shows the population analyses of clone RA<sub>C-14</sub> and of 17 clones obtained by single-cell transplantation from 1 of the 4 clones with 2,500-fold resistance. These 4 clones were derived from RA<sub>C-14</sub>, which had a 1,000-fold resistance. The result indicated that uniformity of cell resistance increases after repeated single-cell inoculation.

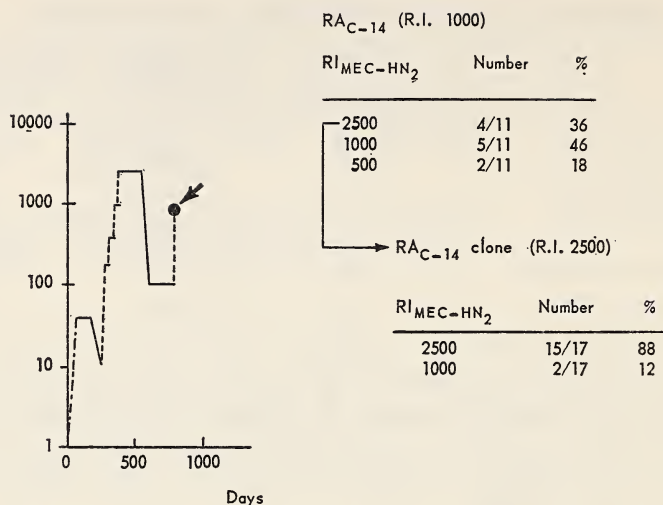
TABLE 21.—Population analysis of control line of Yoshida sarcoma

	Resistance index MEC-HN <sub>2</sub>	Number	Percent
Control	1	13(16)	81
Yoshida sarcoma	2.5	3(16)	19



	R <sub>MEC-HN<sub>2</sub></sub>	Number	%
I.			
RA <sub>C-13</sub> (R.I. 2500)	2500	23(30)	77
	1000	6(30)	20
	250	1(30)	3
II			
RA <sub>C-13</sub> (R.I. 100)	250	6(26)	23
	100	13(26)	50
	50	7(26)	27
III			
RA <sub>C-13</sub> (R.I. 100)	250	2(37)	5
	100	18(37)	49
	50	16(37)	43
	25	1(37)	3

TEXT-FIGURE 14.—Population analyses of resistant lines of Yoshida sarcoma.



TEXT-FIGURE 15.—Population analysis of a single cell clone derived from a single cell clone.

By application of population analysis of the tumor, it was also possible to decrease the resistance index of a line from 100 to 5. The process of this experiment is demonstrated in table 22, in which 37 single-cell clones were prepared from a resistant line, RA<sub>C-13</sub>, with a 100-fold resistance index. One of 37 clones showed a 25-fold resistance, from which 25 clones were prepared after 1-month animal passage. Two of the 25 clones showed a tenfold resistance and were treated similarly (table 22). The lowest index finally reached by this procedure was 5.

Natural decrease of resistance was also observed in the peritoneal cavity of the rat in a certain range but it was possible to effect a rapid decrease by the following treatment:

Yoshida sarcoma, with 5,000-fold resistance to HN<sub>2</sub>, was transplanted into the peritoneal cavity of a rat and 0.025 mg per kg of mitomycin C was injected intraperitoneally every day for 1 month. For the next 2 weeks, 0.05 mg per kg of mitomycin C was similarly injected every day. The tumor was transplanted into a new animal every 5 days. Measurement of resistance of the tumor to HN<sub>2</sub> after 1.5 months showed an MEC of  $1 \times 10^{-2}$  mM, or a resistance of 100-fold, a decrease of one fiftieth from the original resistance (expt. 1). When the same experiments were stopped after 1 month (expts. 2 and 3), the decrease of resistance was one twentieth of the original. During this period, the control group showing a high resistance maintained the original 5,000-fold resistance (table 23).

On the other hand, tumors with a high resistance to HN<sub>2</sub> acquired a fourfold resistance against mitomycin C during the 1 to 1.5 months of treatment. This suggests that the development of resistance, to a second agent was made by the loss of part of the acquired resistance but it has not been possible to alter resistance to HN<sub>2</sub> completely by this procedure.

Based on such observations, similar experiments were carried out with

TABLE 22.—Alteration of resistance index by serial selection of clones of RA<sub>C-13</sub> with a 100-fold resistance index

Resistance index MEC-HN <sub>2</sub>		Number	Percent
1 month	250	2(37)	5
	100	18(37)	49
	50	16(37)	43
	25	1(37)	3
	→ RA <sub>C-13</sub> -Clone: SG 1192-3 (resistance index 25)		
Resistance index MEC-HN <sub>2</sub>		Number	Percent
9 months	50	1(25)	4
	25	22(25)	88
	10	2(25)	8
	→ RA <sub>C-13</sub> -Clone-Clone: SG 1306-88 (resistance index 10)		
Resistance index MEC-HN <sub>2</sub>		Number	Percent
10		13(15)	87
5		2(15)	13

6-mercaptopurine, chloramphenicol, and ethionine, but none of these agents could change the resistance of the tumor cell against HN<sub>2</sub> (table 23).

### CROSSING OF RESISTANCE

The resistance induced by HN<sub>2</sub> could undergo cross-resistance with other nitrogen mustard derivatives and other alkylating agents (table 24).

However, these resistant tumors showed no resistance to colchicine or mitomycin C (table 25 and text-fig. 16), but the tumor with fourfold resistance against mitomycin C by *in vitro* exposure of the control line of Yoshida sarcoma to mitomycin C acquired a fivefold resistance to HN<sub>2</sub>. These observations indicate the complexity of the nature of resistance.

### BIOCHEMICAL OBSERVATIONS OF RESISTANT TUMORS

Respiration and aerobic glycolysis of Yoshida sarcoma were measured with the control line and the one with 5,000-fold resistance to HN<sub>2</sub>. As the results shown in tables 26, 27, and 28 indicate, there was no significant difference between these two tumor strains. Aerobic glycolysis was measured by the manometric method and by chemical determination of lactic acid.

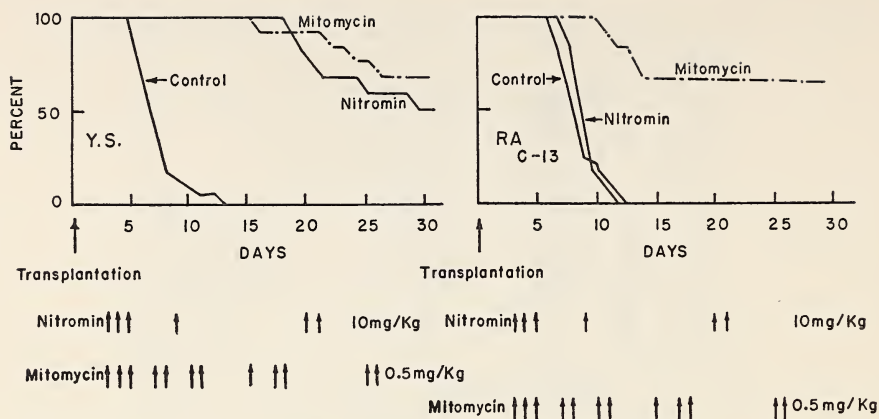
Respiration and aerobic glycolysis of the control and resistant tumors were lowered by the addition of a definite concentration of HN<sub>2</sub>, and the

TABLE 23.—Effect of compounds on resistance to HN<sub>2</sub>

Time (month)	Resistance index MEC-HN <sub>2</sub>							
	RAC-20 Control		RAC-20 Mitomycin injection		RAC-20 6-Mercapto- purine injection		RAC-20 Ethionine injection	
	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II
Start	5,000		5,000		5,000		5,000	
0.5	5,000	5,000	5,000	5,000	5,000	5,000	5,000	5,000
1	5,000	250	5,000	250	5,000	5,000	5,000	5,000
1.5	5,000	100	5,000	100	5,000	5,000	5,000	5,000

TABLE 24.—Cross-resistance to nitrogen mustard derivatives

Compound	Resistance index (MEC) <i>in vitro</i>			Resistance index (IC50) <i>in vitro</i>		
	Yoshida sarcoma	RAc-7	RC	Yoshida sarcoma	RAc-7	RC
$\text{CH}_3\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$	$\frac{1}{(1 \times 10^{-4} \text{ mm})}$	1,000	10	$\frac{1}{(1.2 \times 10^{-5} \text{ mm})}$	2,000	10
$\text{C}_2\text{H}_5\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$	$\frac{1}{(2.5 \times 10^{-3} \text{ mm})}$	40				
$\text{NCCH}_2\text{CH}_2\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$	$\frac{1}{(1 \times 10^{-3} \text{ mm})}$	25	10	$\frac{1}{(1.2 \times 10^{-4} \text{ mm})}$	20	10
$\text{C}_6\text{H}_5\text{N}(\text{CH}_2\text{CH}_2\text{OSO}_2\text{C}_6\text{H}_4\text{CH}_3)_2$	$\frac{1}{(5 \times 10^{-4} \text{ mm})}$	10				
$\text{HN}(\text{CH}_2\text{CHClCH}_3)_2$	$\frac{1}{(2.5 \times 10^{-3} \text{ mm})}$	4				
$\text{H}_2\text{NOCCH}_2\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$				$\frac{1}{(1.2 \times 10^{-4} \text{ mm})}$	20	5
$(\text{C}_2\text{H}_5)_2\text{N}(\text{CH}_2)_4\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$				$\frac{1}{(8.8 \times 10^{-5} \text{ mm})}$	10	5

TEXT-FIGURE 16.—Non-cross-resistance to mitomycin C of  $\text{HN}_2$ -resistant tumor.TABLE 25.—Resistance indices to  $\text{HN}_2$ , mitomycin C, and colchicine

	Yoshida sarcoma	RA <sub>C-13</sub>	RA <sub>C-20</sub>
$\text{CH}_3\text{N} \begin{cases} \text{CH}_2\text{CH}_2\text{Cl} \\ \text{CH}_2\text{CH}_2\text{Cl} \end{cases}$	1*	2,500	10,000
Mitomycin C	1†	1	1
Colchicine	1‡	1	1

\*MEC:  $1 \times 10^{-4}$  mM†MEC: 0.0025  $\mu\text{g}/\text{ml}$ ‡MEC: 0.25  $\mu\text{g}/\text{ml}$ 

rate of lowering according to  $\text{HN}_2$  concentration was also similar between the resistant and control lines (tables 29 and 30).

Enzyme activity of the control and the resistant lines was examined by the measurement of catalase, cholinesterase, transaminase (GOT and GPT), alkali and acid phosphatase,  $\beta$ -glucuronidase, glutaminase, asparaginase, xanthine oxidase, lactic dehydrogenase, cytochrome *c* oxidase, phosphorylase, hexokinase, glucose-6-phosphatase, sulfatase, and pyruvic

TABLE 26.—Endogenous respiration of Yoshida sarcoma\*

$\text{QO}_2/10^7$ cells		
Control strain	Resistant strain (5,000-fold)	
16.5	16.8	
17.4	21.5	
12.9	17.2	
26.2		
Mean	18.2	18.5

\*Conditions: Krebs-Ringer phosphate buffer, pH 6.8;  $2-4 \times 10^7$  cells per 2 ml.

TABLE 27.—Aerobic glycolysis of Yoshida sarcoma

$Q_{O_2}/10^7$ cells		
Control strain	Resistant strain (5,000-fold)	
63.4	72.1	
73.7	50.3	
64.5		
Mean	67.2	61.2

\*Conditions: Krebs-Ringer phosphate buffer contained 0.2 percent glucose, pH 6.8;  $2-4 \times 10^7$  cells per 2.2 ml;  $Q_{M^{0.2}}$  per  $10^7$  cells.

TABLE 28.—Lactic acid (formations of Yoshida sarcoma)

Lactic acid ( $\mu\text{g/hr}/10^7$ cells)		
Control strain	Resistant strain (5,000-fold)	
193	162	
174	217	
184	222	
Mean	187	200

\*Conditions: Krebs-Ringer phosphate buffer contained 0.2 percent glucose, pH 6.8;  $3-5 \times 10^7$  cells per 2.2 ml.

TABLE 29.—Effect of  $\text{HN}_2$  on endogenous respiration of Yoshida sarcoma

$\text{HN}_2(\text{mm})$	$Q_{O_2}/10^7$ cells	
	Control strain	Resistant strain
0	26.2	17.2
1	20.8	16.2
2.5	18.4	15.7
5	14.6	12.8
10	9.8	9.3

TABLE 30.—Effect of  $\text{HN}_2$  on aerobic glycolysis of Yoshida sarcoma

$\text{HN}_2(\text{mm})$	Lactic acid ( $\mu\text{g/hr}/10^7$ cells)	
	Control strain	Resistant strain (5,000-fold)
0	184	162
1	65.8	65.8
2.5	42.3	35.2
5	23.5	23.5
10	9.4	11.7

TABLE 31.—Identification of oligosaccharides

		Sample		Maltose	
R <sub>F</sub> Value (BuOH: Pyridine: H <sub>2</sub> O) 6 : 4 : 3		0. 28, 0. 21, 0. 13, 0. 0		0. 28	
Hydrolysis	Infrared absorption (cm <sup>-1</sup> )	Glucose		Glucose	
		1040	1250	1040	1250
Octa acetate	Elemental analysis	1375	1760	1375	1760
		C: 49. 50 H: 5. 76		C: 49. 56 H: 5. 64	

dehydrogenase activities. There was no significant difference between the two strains.

When the cells were stained with periodic acid-Schiff (PAS) reagent according to the method of Lillie (18), the number and type of PAS-positive cells of HN<sub>2</sub>-resistant lines seemed markedly higher than those of the control Yoshida sarcoma cells. The presence of oligosaccharides in the cells of resistant lines of Yoshida sarcoma, showing 4 kinds of glucose polymers, was demonstrated by paper chromatography. The principal constituent was identified as maltose (table 31).

Glycogen and oligosaccharides were determined in both the control and resistant lines of Yoshida sarcoma. There was a higher concentration of both components in the cells of the resistant lines (table 32). However, these components increased in the cells of the control line of Yoshida sarcoma when treated with nitrogen mustard derivatives. Table 33 shows the increase of glycogen and oligosaccharides that occurred 24 hours after a single injection of Nitromin with a decrease again after 72 hours. The change was therefore reversible, but by repeated exposure to the agent this change seemed to fix irreversibly when there was resistance to chemicals.

## CONCLUSION

Tissue culture of tumor cells provides a suitable method for screening antineoplastic compounds for preliminary evaluation of their cytotoxic action on tumor cells.

In our laboratory, we have chiefly used rats bearing ascites tumors, Yoshida sarcoma or ascites hepatomas, induced by azo dyes, as the

TABLE 32.—Glycogen and oligosaccharides in nitrogen-mustard-resistant lines of Yoshida sarcoma

Line	Resistance index MEC	Glycogen*	Oligosaccharides*
Yoshida Sarcoma	1	0. 34	0. 2
RA <sub>C-13</sub>	100	0. 40	0. 2
RA <sub>C-20</sub>	100	2. 0	0. 2
RA <sub>C-21</sub>	2,500	8. 7	1. 4
RA <sub>C-22</sub>	5,000	3. 3	1. 2

\*mg per g as glucose.

TABLE 33.—Effects of Nitromin on Yoshida sarcoma, in rats given 10 mg per kg intraperitoneally

Hours after injection	Ascites		Tumor	
	CE*	PAS	Glycogen†	Oligosaccharides†
0 (control)	—	—	0.1	0.2
24	±	+	0.7	0.2
48	+	+++	2.4	1.0
72	+	++	0.2	0.3

\*Cytological effect.

†mg per g as glucose.

screening tools. The antitumor effect observed in these animal experiments should be understood as the result of drug action on the tumor cells under the mutual influence of host and tumor. Thus, there was a need to determine the effect of compounds on tumor cells alone, without the influence of the host, in order to compare the results of both *in vivo* and *in vitro* screening with the same tumor. For this purpose, a method of primary cell culture of the ascites tumors, used presently in our laboratory for the *in vivo* screening, and the technique of *in vitro* screening were established. Combined screening of one compound gave far more information than the *in vivo* test alone, such as the mechanism of action, stability *in vivo*, and reaction rate of the test compounds—especially the conditions and mechanism of activation of the compounds with latent activity by the tumor cells themselves. The cell population of the ascites tumors did not seem to have a uniform constitution originally in their sensitivity to chemicals, and often this mosaic feature of the cell population changed during serial cultivation for a long period *in vitro*. Therefore, the use of primary cell culture, starting with a rather large cell inoculum, is recommended for this kind of investigation.

The problem of acquired drug resistance of Yoshida sarcoma could also be analyzed well with this technique of cell culture. Induction of resistance and quantitative determination of the resistance index were carried out, without the influence of the condition of the host. The resistance index of a tumor with an extremely high resistance could not be determined without the *in vitro* technique, because such a high dose of the chemical agent was necessary to affect the resistant tumor cells that the host animal died from toxicity.

The results of the analyses of resistant lines of Yoshida sarcoma, induced and assayed *in vitro* and analyzed with single cell clones, were as follows: 1) Although the drug sensitivity of the control or resistant lines of Yoshida sarcoma is expressed as a whole cell population, population analyses of the clones suggest that the resistance of each of the constituting cells seemed to be distributed in a certain range. 2) This distribution, however, is not so great as to undergo crossing between a nonresistant cell and a 1,000-fold resistant cell, and development of resistance cannot be explained merely by the shift in composition of the constituent cells by selection, owing to the action of chemicals. 3) It may be that the decrease in sensitivity of

tumor cells to chemicals, by the foregoing procedure, is caused by mutagenic changes in the cells from exposure to an agent such as nitrogen mustard—a well-known chemical mutagen—that decreased the drug sensitivity. On the other hand, a single-cell clone derived from cells of a single-cell clone also had a varied sensitivity to chemicals and the repeated procedure of selecting the least resistant clone among single-cell clones, prepared from a cell population of a resistant tumor, decreased step by step their resistance index. So perhaps even the cells in a single-cell clone tend to have varied sensitivity, which is distributed within a small range during proliferation in the peritoneal cavity of rats. Thus, exposure to the drug killed the sensitive cells and thereby spontaneously deviated the selection, or picking up of a small number of cells, to less sensitive cells during proliferation. By numerous repetition of this procedure—spontaneous deviation and selection by chemicals—the resistance index might increase stepwise up to a high value without presuming a mutagenic change by chemical agents.

In the present stage of this experimental study, it can be concluded that the induction of high resistance to Yoshida sarcoma cells by this *in vitro* technique might be possibly due to mutagenic change and to spontaneous deviation of the sensitivity of tumor cells during proliferation, even in natural circumstances.

## REFERENCES

- (1) LETTRÉ, H., and SCHLEICH, A.: Untersuchungen am Yoshida-tumors. *Naturwissenschaften* 41: 505, 1954.
- (2) SCHLEICH, A.: Wachstum einzelner explantierter zellen des Yoshida-tumors. *Naturwissenschaften* 42: 50, 1955.
- (3) ISHIDATE, M., SAKURAI, Y., IMAMURA, H., and MORIWAKI, A.: Studies on carcinostatic substances. XX. Studies on the culture of Yoshida sarcoma cells *in vitro*. *Chem Pharm Bull* 7: 690-694, 1959.
- (4) ———: Studies on carcinostatic substances. XXII. Screening method for antimitotic substance using the *in vitro*-cultured Yoshida sarcoma cells. *Chem Pharm Bull* 7: 873-877, 1959.
- (5) MORIWAKI, A.: Studies on carcinostatic substances. XI. Application of cell counting method to the screening of antitumor substances using the *in vitro*-cultured Yoshida sarcoma cells. *Chem Pharm Bull* 10: 462-467, 1962.
- (6) KATSUTA, H., TAKAOKA, T., HORI, M., OKOMURA, H., YASUKAWA, M., SAITO, S., and SUZUKI, S.: Cultivation of rat ascites hepatoma cells in the simplified replicate tissue culture. *Jap J Exp Med* 27: 443-458, 1957.
- (7) TAKAOKA, T.: Fluid-suspension culture of rat ascites hepatoma cells and tissue culture strain cells. *Jap J Exp Med* 28: 381-393, 1958.
- (8) SAKURAI, Y., MORIWAKI, A., and TONOSAKI, H.: Primary cell-culture of rat ascites hepatoma as a tool for antitumor screening. *Gann* 54: 217-220, 1963.
- (9) HIRONO, I., and YOKOYAMA, C.: Development of the resistance of tumor in chemotherapy. *Gann* 45: 496-498, 1954.
- (10) ISHIDATE, M., SAKURAI, Y., IMAMURA, H., and MORIWAKI, A.: Studies on carcinostatic substances. XXXII. Acquired resistance of Yoshida sarcoma by treatment with derivatives of nitrogen mustard. *Chem Pharm Bull* 8: 1052-1053, 1960.

- (11) SAKURAI, Y., and MORIWAKI, A.: Occurrence of drug resistance during experimental therapy of rats bearing Yoshida sarcoma with nitrogen mustard derivatives. *Gann* 54: 379-380, 1963.
- (12) YOSHIDA, T.: Yoshida Nikushu (Yoshida sarcoma). Nara Shobo, Japan, 1949.
- (13) ISHIDATE, M., SAKURAI, Y., YOSHIDA, T., SATOH, H., and IMAMURA, H.: Experimental studies on chemotherapy of malignant growth employing Yoshida sarcoma animals. V. Evaluation and comparison of curative effects of compounds tested using the diagram of "percent survival." *Gann* 45: 484-488, 1954.
- (14) ISHIDATE, M., SAKURAI, Y., IMAMURA, H., and MORIWAKI, A.: Studies on carcinostatic substances. XXVII. Antitumor activity of 2-chloroethylamine derivatives on the *in vitro*-cultured Yoshida sarcoma cells. *Chem Pharm Bull* 8: 444-448, 1960.
- (15) IMAMURA, H.: Studies on carcinostatic substances. XXVIII. Activation of the derivatives of 2-chloroethylamine with latent activity. *Chem Pharm Bull* 8: 450-454, 1960.
- (16) SAKURAI, Y., and MORIWAKI, A.: The *in vitro* induction of drug resistance of Yoshida sarcoma to alkylating agents. *Gann* 54: 473-479, 1963.
- (17) MORIWAKI, A.: Determination of resistance index of tumor cells to antitumor agents. *Gann* 54: 323-329, 1963.
- (18) LILLIE, R. D.: Histochemical comparison of the Casella Bauer and periodic acid oxidation-Schiff leucofuchsin technics. *Stain Techn* 26: 123-136, 1951.



## Cancer Metastasis and Ascites Tumors

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**M**ETASTASIS is one of the most troublesome problems in cancer. Even in patients in which cancer is found and diagnosed in the early stages and surgical operation is possible, no physician can tell whether a complete cure can be effected. Whether there might be any recurrence or metastasis, when it might occur, and where the metastasis might be formed are difficult to predict. As Yoshida (1) has said, the treatment of cancer would be much simpler if the malignant growth was incapable of metastasizing.

In our laboratory we have been studying the problem of cancer metastasis, especially the mechanism of metastatic formation through the use of animal tumors such as Yoshida sarcoma and ascites hepatomas in rats and mice (2). This paper deals with some of the problems we investigated, in which we used these ascites tumors. A series of experiments, in which ascites hepatomas in C3H mice (3) were used, served as the model for cancer metastasis following surgery. These tumors are suitable because they possess the ability to metastasize to the lymph nodes, lungs, or to other organs and tissues of the body (4). Thus, by implanting tumor cells in the subcutaneous caudal tissues in mice, lymph node metastasis can be investigated.

The course of metastatic development requires several steps: 1) liberation of tumor cells from the primary lesion; 2) movement of the liberated cells into tissue spaces around the blood and lymph vessels; 3) invasion of the cells into the vessels; 4) migration of the cells throughout the vascular system; 5) lodgment of the cells in organs and tissues; and 6) proliferation of the cells to form a metastatic focus.

The fourth step in this process involves the dissemination of cancer cells by the circulating blood. The correlation between metastasis and tumor cells in the blood was investigated experimentally.

Our results suggest that chemotherapy might be effective in inhibiting the growth of disseminated cancer cells, especially when there are few, and might prevent the development of metastasis. Some basic results were also obtained regarding host-conditioning factors that may control the growth of small numbers of cancer cells.

## MATERIALS FOR THE EXPERIMENTAL STUDY OF METASTASIS

To study the mechanism of metastasis experimentally, adequate materials which produce marked and frequent metastasis are required—many spontaneous tumors and tumors induced by carcinogens are available. Although the frequency of metastasis can be studied with these tumors, they are not adequate for an investigation of the mechanism of metastatic formation, because of the lack of uniformity as to site of metastasis and relative infrequency thereof. Odashima (5) used azo dyes to induce primary liver cancers in 299 rats and found that 101 (34%) developed metastases to the lungs and that 31 (10%) developed metastases to the mediastinal lymph nodes (table 1).

TABLE 1.—Metastases in rats with primary hepatomas induced by  
azo dyes

Number of animals examined	Animals with metastases	
	In lungs	In mediastinal lymph nodes
299	101 (34%)	31 (10%)

In our laboratory, we studied the frequency of metastasis in inbred C3H female mice with spontaneous mammary cancers. In 1957–58 we found that 4 of 32 (13%) mice developed metastases to the lymph nodes and lungs (6) and in 1960–61 that 19 of 57 (33%) mice developed metastases to the lungs, lymph nodes, or both (table 2). Thus we see that tumors of the same kind sometimes metastasized but others did not. In further experiments, primary tumors from animals with metastases and from those with no metastases were transplanted into other C3H mice. The occurrence or absence of metastasis in the animals receiving the transplanted tumors showed no correlation whatsoever with any metastatic findings in the animals from which the tumors were taken.

TABLE 2.—Metastases in spontaneous mammary tumors in C3H mice

Number of mice examined	Number of animals		
	With metastases	Without metastases	No information
57	19 (33%)	36 (63%)	2
	Location { lymph nodes lungs	14 10	

Nevertheless, transplantable tumors are often used in experiments on metastasis. Many established, transplantable tumors have been reported, but the metastatic characteristics have been investigated in detail only in a few cases. From the data in a report on transplantable and transmissible animal tumors by Dunham and Stewart (7), we calculated that

of 243 tumors, metastases occurred in only 40 percent; lymphomas and leukemias seemed to metastasize more frequently than carcinomas and sarcomas (table 3). From another report by Stewart *et al.* (8), we estimated that in 50 tumors metastases was fairly frequent in 9 and less frequent in 24 (table 4).

TABLE 3.—Frequency of metastases with transplantable tumors\*

Tumors	Number of tumors	Metastases†			
		Frequent	Rare	None	No information
Epithelial	101	29 (29)	16 (16)	28 (28)	28 (28)
Lymphoma and leukemia	60	54 (90)	2 (3)	0	4 (7)
Nonepithelial	68	11 (16)	11 (16)	28 (41)	18 (27)
Others	14	4 (29)	0	10 (71)	0
Total	243	98 (40)	29 (12)	66 (27)	50 (21)

\*From a survey by Dunham and Stewart (7).

†Figures in parentheses indicate percent.

TABLE 4.—Frequency of metastases with transplantable tumors\*

Tumor	Number of tumors	Metastases†			
		Frequent	Rare	Negative	No information
Epithelial	18	3 (16.7)	6 (33.3)	3 (16.7)	6 (33.3)
Nonepithelial	14	3 (21.4)	6 (42.8)	3 (21.4)	2 (14.6)
Leukemia	5	1 (20.0)	4 (80.0)	0	0
Others	13	2 (15.4)	8 (61.5)	1 (7.7)	2 (15.4)
Total	50	9 (18.0)	24 (48.0)	7 (14.0)	10 (20.0)

\*From a survey by Stewart *et al.* (8).

†Figures in parentheses indicate percent.

Tumors used in these experiments were: Ehrlich ascites carcinoma, Krebs 2 carcinoma, Crocker sarcoma 180, and Sarcoma 37 in mice; Murphy-Sturm lymphosarcoma and Yoshida sarcoma in rats; Brown-Pearce carcinoma and V2-carcinoma in rabbits, etc. In addition, Ketcham *et al.* (9) reported frequent lung metastases with Cloudman melanoma S91, S91A amelanotic melanoma, Osteogenic sarcoma 112, Lewis sarcoma T241, and Sarcoma dba49 transplanted into the thighs of mice. However, tumors rather consistently producing metastasis are extremely rare.

Since it is often difficult to cause experimental tumors to metastasize, a technique of "artificial metastasis," in which tumor cells are injected into the blood vessels and tumor development in the lungs and other areas of the body is observed, has been employed by many investigators. However, we wanted experimental tumors in which the metastasis occurred naturally and frequently. Three strains of mouse ascites hepatomas (MH 134, MH 129F, and MH 129P) were transplanted into host animals such as the C3H/HeN inbred mice and (C3H/HeN ♂ × dd ♀)F<sub>1</sub> hybrid

mice, and uniform "takes" were usually obtained. The animals died without showing any spontaneous regression of tumors transplanted via any route of inoculation (table 5), although there were a few animals dying with tumors which showed the "unusual" procedure (10) for tumor growth.

TABLE 5.—Transplantability of MH 134, MH 129F, and MH 129P mouse ascites hepatomas in C3H inbred and (C3H × dd)F<sub>1</sub> hybrids

Site of transplantation	Effective transplan- tation (No. of mice)	Died		Deaths (%)	Average survival period (days)
		Normal course	Abnormal course		
Intraperitoneal	862	855	7	100	16
Subcutaneous	243	241	2	100	35
Intravenous	53	53	0	100	28
Tail	62	62	0	100	39
Total	1, 220	1, 211	9	100	

Besides these three mouse ascites hepatomas, various ascites tumor strains such as Yoshida sarcoma and Yoshida ascites hepatoma of rats and other mouse ascites tumors have been used.

In the ascites tumors, single cells or clumps of cells grow suspended in the fluid without having any stromal tissue, such as connective tissue. This ability to live and proliferate in a fluid medium is characteristic of ascites tumors and is considered as an essential factor in the initiation of the metastatic process.

## RECURRENCE AND METASTASIS OF MOUSE ASCITES HEPATOMAS

When mouse ascites hepatomas were subcutaneously transplanted in the backs of C3H mice, tumors developed and all the animals died (fig. 1). If the tumors were surgically removed at certain intervals after the inoculation, the animals usually survived longer, but recurrence, or metastasis, or both (fig. 2) were very frequent and almost inevitable when the operation was not performed until 2 weeks after transplantation (figs. 3 and 4 and table 6). Metastasis was observed in lymph nodes, lungs, etc. (tables 7 and 8). The rate of lymph node metastasis was highest for the MH 134 tumor (74% in the untreated control group, and 84% in those treated surgically) and less frequent for the MH 129P tumor. Local recurrence of tumors at the sites of surgical excision was almost 100 percent in the MH 129P group and 50 percent in the MH 134 group. The frequency of metastatic formation at various sites for the MH 129F tumor was roughly intermediate between the frequencies for MH 129P and MH 134. Thus, each tumor seems to have its own metastasizing characteristics. The concept of individuality in the malignancy

of the rat ascites hepatomas has been stated (11), and the different characteristics of each tumor are now being investigated biologically and morphologically. The foregoing results seem to support this concept.

TABLE 6.—Recurrence and metastases following surgical removal of subcutaneously transplanted tumors MH 134, MH 129F, and MH 129P

Treatment	Number of mice	Died from tumor	Cured	
			Number	Percent
Control, untreated	46	46	0	0
Operation 2 weeks after transplantation	103	102	1	1
Operation 1 week after transplantation	57	26	31	54

TABLE 7.—Frequency of metastases in C3H mice in which mouse ascites hepatomas MH 134, MH 129F, or MH 129P were transplanted subcutaneously

Site	Frequency of metastases (%)			
	MH 134	MH 129F	MH 129P	Total
(Local)	100	100	100	100
Lymph node	74	73	13	54
Lung	73	60	33	57
Heart	36	50	18	34
Spleen	50	30	0	29
Number of mice examined	19	11	15	45

TABLE 8.—Frequency of recurrence and metastases in C3H mice in which the subcutaneously transplanted tumors were excised 2 weeks after inoculation

Site	Frequency of metastases (%)			
	MH 134	MH 129F	MH 129P	Total
Local	50	70	97	73
Lymph node	84	53	24	54
Lung	96	79	81	85
Heart	43	36	26	34
Spleen	61	21	0	26
Liver	10	0	13	8
Number of mice examined	32	30	33	95

A series of model experiments was carried out to evaluate the effect of cancer chemotherapy after surgery to prevent recurrence and metastasis (11, 12). Table 9 shows that surgery alone is not always adequate. In 15 of 100 animals administered Nitromin (nitrogen mustard *N*-oxide) after surgery, there was survival without any signs of recurrence or metastasis. When surgery alone was performed 1 week after inoculation, about 54 percent of the animals were cured, but the rate was higher for the group in which surgery was followed by chemotherapy. The role of cancer chemotherapy will be discussed in detail later.

TABLE 9.—Effect of chemotherapy after surgery on recurrence and/or metastasis of subcutaneously transplanted ascites hepatomas in mice.

Treatment	Number of mice	Died from tumor	Cured	
			Number	Percent
Control, untreated	46	46	0	0
Operation 2 weeks after transplantation	103	102	1	1
Operation 2 weeks after transplantation, followed by chemotherapy	100	85	15	15
Operation 1 week after transplantation	57	26	31	54
Operation 1 week after transplantation, followed by chemotherapy	81	29	52	64

## EXPERIMENTS ON LYMPH NODE METASTASIS

Lymph node metastasis of malignant tumors in humans is so common that it is seen in almost all autopsies, but it is difficult to induce with experimental tumors in animals. Koch (13) used "Ehrlich" carcinoma from lymph node metastasis in mice, performed serial inoculations, and obtained a tumor strain which metastasized with a high degree of frequency. Subsequently, other investigators used this "Ehrlich ascites cancer" but did not obtain such a high frequency of metastatic formation.

Lymph node metastasis was frequently observed with mouse ascites hepatomas transplanted subcutaneously in the back. If the tumor cells were inoculated selectively in particular sites such as the subcutaneous caudal lymph spaces (fig. 5), metastasis was induced in the regional lymph nodes (14, 15). In almost 100 percent of (strain C3H) mice inoculated, tumors formed and the mice died within 1½ months after transplantation. The tumor growth usually spread to the proximal part of the tail and, almost without exception, the metastasis involved the sciatic, inguinal, and retroperitoneal lymph nodes (table 10).

TABLE 10.—Sites and frequency of metastases in mice with caudal transplants of mouse ascites hepatomas

Tumor	Number of animals with metastases (%)			
	MH 134	MH 129F	MH 129P	Total
Retroperitoneal lymph nodes *	41 (93)	16 (94)	16 (100)	73 (95)
Superficial lymph nodes†	25 (57)	10 (59)	8 (50)	43 (56)
Lung	29 (66)	16 (94)	11 (69)	56 (74)
Heart	7 (16)	4 (24)	2 (13)	13 (17)
Kidney	7 (16)	2 (12)	3 (19)	12 (16)
Adrenals	7 (16)	3 (18)	6 (38)	16 (21)
Others ‡	12 (27)	8 (47)	8 (50)	28 (36)
Number of mice examined	44	17	16	77

\* Includes the lumbar, caudal, and renal nodes.

† Includes the inguinal and axillary nodes.

‡ Includes the spleen, bone marrow, brain, and genital organs.

Experiments were performed to determine the time at which metastasis occurred. The tails were amputated at an early stage, such as 5, 10, and 15 days after inoculation, and the animals were kept under observation. A number of animals died as a result of metastasis as seen in figures 6, 7, and 8 and, as reported previously, it was assumed that the tumor cells had already been migrating from the primary site of growth in the tail to the main part of the body, at the time of amputation.

In another experiment, sciatic lymph nodes, the primary nodes in the tail area, were removed at various intervals after caudal transplantation and examined for tumor cells (table 11). The histologic examination of the cells was supplemented by biologic methods of inspection, *i.e.*, the lymph nodes were minced with scissors and then implanted into the peritoneal cavity of another mouse. The presence of proliferating tumor cells in the ascites indicated that metastasis had occurred in the lymph nodes. Thus, tumor cells not detected in the lymph nodes by histologic methods until 15 days or more after caudal transplantation were found earlier by biologic methods. This was also demonstrated in another experiment, in which the tumors were inoculated caudally as described earlier. The tails were amputated at certain intervals after transplantation and at the same time the sciatic nodes were removed surgically and examined histologically for metastasis. Animals in which no metastasis was detected at this time were kept under observation and examined for recurrence, or metastasis, or both. About half of the animals in the group in which the tails were amputated 15 days after transplantation died with metastasis, in spite of the absence of metastasis in the regional lymph nodes at the time of amputation. Even those in which the tails were amputated as early as 5 to 7 days after transplantation had recurrence, or metastasis, or both, in about one fourth of the mice, as shown in table 12.

TABLE 11.—Detection of tumor cells in the sciatic lymph nodes at various intervals following caudal inoculation of MH 134 tumor

Removal of lymph nodes (days after inoculation)	Histologic examination		Biologic examination*	
	Number of mice	Percent	Number of mice	Percent
5	0/20†	0	0/10	0
7	1/17	6.0	4/10	40
10	0/9	0	7/10	70
15	7/10	70	—	—

\*The tissues of the lymph nodes were pulped and transplanted into the peritoneal cavity of a group of mice, and the ascitic fluid of the recipients examined for proliferation of tumor cells.

†Number of mice with metastasis/number of mice examined.

In human surgical interventions, it is common to remove regional lymph nodes to prevent the spread of malignant growth. A series of experiments was designed to examine the effectiveness thereof for prevention of the recurrence or metastasis in animals. MH 134 and MH 129F tumors were transplanted into the tail in the same manner as before.

In the control group, the tails were amputated, while in the experimental group the amputation of the tails and the removal of the regional (sciatic) lymph nodes were carried out at various intervals after transplantation. The metastatic formation was carefully examined at death or when sacrificed 2 months after transplantation. As shown in table 13, the frequency of metastasis, or recurrence, or both, was lower in the experimental groups than in the controls. These results also indicate that recurrence or metastasis cannot be prevented solely by the removal of the regional lymph nodes. The prevention of recurrence and metastasis seems to be correlated with the time from transplantation to surgical intervention. As is well known, removal of the regional lymph nodes reduces the frequency of recurrence and metastasis when surgery to excise the primary site of tumor growth is carried out at an early stage. Complete cure could not be achieved by surgical intervention alone.

TABLE 12.—Metastases in mice in which the tails were amputated at various periods after inoculation of MH 134 or MH 129F tumors and in which the sciatic lymph nodes showed no metastases histologically

Amputation (days)	Number of mice examined	Number of mice in which metastases developed later*
5	34	8 (24)
7	28	7 (25)
10	17	8 (47)
15	6	3 (50)

\*Figures in parentheses indicate percent.

TABLE 13.—Effect of removal of regional lymph nodes on prevention of recurrence and metastasis in mice in which the tumor-bearing tails were amputated at various periods after inoculation of MH 134 or MH 129F tumors

Amputation (days)	Control mice with amputation	Mice with amputation and removal of regional lymph nodes
5	18/39* (46) †	10/37 (27)
7	14/38 (37)	10/37 (27)
10	13/20 (65)	9/20 (45)
15	15/20 (75)	11/18 (61)

\*Number of mice with metastasis/number of mice examined.

†Figures in parentheses indicate percent.

Metastasis may be revealed early in some cases of cancer of the stomach and other organs in humans or not until late in others. Thus it is assumed that there is a difference in the speed of metastasis. This was clearly demonstrated by an experiment in which mouse ascites hepatomas MH 129F, MH 129P, and FM3A [(16) an ascites tumor of mammary cancer origin in C3H mice] were transplanted into the tails of C3H mice.

The metastasis in the sciatic lymph nodes was serially examined after transplantation; that there was a marked difference in each tumor strain is shown in table 14. When the mice were autopsied, the frequency of metastasis did not differ greatly for each tumor; almost all the animals died with metastatic formations. These results showed that the MH 129F and the MH 134 tumors were similar in their ability to metastasize into lymph nodes, but metastases occurred more slowly with MH 129P. A similar result was obtained in a previous experiment in which MH 129P was transplanted subcutaneously in the back. After excision of the tumor, marked metastasis was somewhat more frequent in the areas of hematogenous dissemination, such as the lungs, than in the lymph nodes. This also indicates a difference in the speed of metastasis which can be considered a characteristic of tumor growth.

TABLE 14.—Histologic detection of metastasis to sciatic lymph nodes at various periods following caudal inoculation of mouse ascites tumors

Days after inoculation	MH 134	MH 129F	MH 129P	FM3A
1-2	4/49* ( 8.1)†	—	—	—
3-5	3/49 ( 6.1)	3/19 (15.8)	0/7 ( 0 )	0/10 ( 0 )
7-10	12/46 (26.0)	7/30 (23.3)	0/18 ( 0 )	2/18 (11)
15	7/10 (70.0)	6/10 (60.0)	0/20 ( 0 )	9/20 (45)
25	—	—	6/10 (60.0)	6/10 (60)

\*Number of mice with metastasis/number of animals examined.

†Figures in parentheses indicate percent.

## TUMOR CELLS IN THE BLOOD OF TUMOR-BEARING ANIMALS WITH METASTASES

Many times cancer recurs several years after surgery or exhibits delayed metastasis despite the absence of any metastasis at the time of surgery. This phenomenon can be interpreted as follows: Probably the few cancer cells remaining in the body had not proliferated enough to be detected by known techniques. In pathology, this concept that the growth of cancer is initiated by the presence of cancer cells, although a few animal tumors of viral origin are exceptions, is current.

As described previously, the mouse ascites hepatomas have marked metastasizing ability. When the subcutaneously transplanted tumors, for example, were removed surgically 2 weeks after inoculation, almost all the animals died from recurrence, or metastasis, or both. When the primary tumors were excised no enlargement of the lymph nodes could be observed. A number of animals in the group were sacrificed and the lungs and lymph nodes examined histologically, but often hardly any metastatic tumor cells were found. When the lungs and lymph nodes removed from the donor animals were minced and inoculated into the peritoneal cavities of the recipient mice, however, proliferation of tumor cells in the ascites was observed. Thus, the existence of tumor

cells in the lungs and lymph nodes was demonstrated in about 75 percent of the animals examined 15 days after inoculation (2).

Furthermore, it was found that sometimes the tumor cells appeared in the circulating blood early in tumor growth (17, 18). The circulating blood of the mice transplanted with MH 134 was examined for tumor cells and, as seen in table 15, the longer the interval after inoculation the more frequently tumor cells were detected in the blood. Then, the animals were killed and examined for emboli and/or growth of tumor cells in the lungs, as well as for lymph nodes metastasis. Metastatic growth of tumors and tumor emboli were rarely observed in the lungs within the first 10 days after transplantation. Tumor emboli in the lungs increased gradually and there was a marked increase in metastatic growth between 21 and 30 days after inoculation. In the animals in which tumor cells were not detected in the circulating blood, no metastatic growth was observed in the lungs, even 21 to 30 days after inoculation. Lymph nodes metastasis was observed frequently in animals whose blood was positive for tumor cells, but it was also found occasionally in those whose blood was negative for tumor cells (table 16).

TABLE 15.—Appearance of tumor cells in the blood of C3H mice after subcutaneous transplantation of MH 134 tumor

	Days after transplantation				Total
	1-5	5-15	16-25	26-35	
Number of mice examined	11	50	34	10	105
Number with blood positive for tumor cells	0	13	22	8	43
Percentage with blood positive for tumor cells	0	26	65	80	41

TABLE 16.—Correlation between presence of tumor cells in the blood and development of metastases in the lungs of mice with MH 134 tumor

Period after inoculation (days)	Presence of tumor cells in the blood	Number of mice examined	Lung metastases		Lymph-node metastases
			Emboli	Growth	
1-10	+	11	1	1	2/8
11-20	+	18	10	1	7/18
21-30	+	8	7	6	6/8
1-10	—	33	0	0	0/27
11-20	—	23	1	0	4/23
21-30	—	2	0	0	0/2

In another series of experiments, the correlation between the appearance of tumor cells in the circulating blood of rats and the frequency of metastasis was investigated. The appearance of tumor cells in the heart blood was examined serially after various strains of the Yoshida rat ascites

hepatoma and the Yoshida sarcoma were transplanted intraperitoneally. As seen in table 17, tumor cells appeared early in the blood of rats in which certain tumor strains had been transplanted and later in animals bearing other tumor strains; in some no tumor cells appeared in the blood. The lung metastases of these animals were examined histologically: The animals in which tumor cells were in the blood had a high frequency of metastasis, but those with no tumor cells had a low frequency (19, 20).

From the results shown in tables 16 and 18, it seems reasonable to conclude that the appearance of tumor cells in the blood is an indication of the presence of metastatic lesions or, at least, of the forthcoming development of metastases.

TABLE 17.—Detection of tumor cells in blood of rats with intraperitoneally inoculated ascites hepatomas

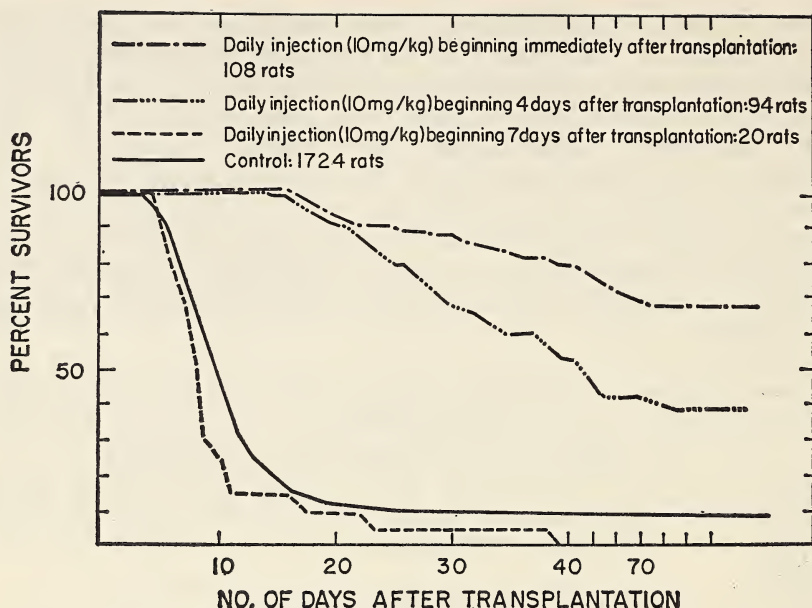
Type of tumor	Presence of tumor cells (days after transplantation)																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
AH 13	—	—	+	+	+	+	+										
AH 66F	—	—	—	—	+	+	+	+									
AH 130	—	—	—	—	—	+	—	—	—	+	—	—	—				
AH 21	—	—	—	—	—	—	+	+									
AH 7974	—	—	—	—	—	—	—	+	—	—	—	—	—				
AH 149	—	—	—	—	—	—	—	—	+	—	—	—	—	+	+	—	+
AH 99	—	—	—	—	—	—	—	—	—	+							
AH 49	—	—	—	—	—	—	—	—	—		+	—	—				
AH 63	—	—	—	—	—	—	—	—	—	—	—	—	+	—			
AH 39	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+		
YS, intra-peritoneal	—	—	—	+	+	+											
YS, subcutaneous			—		+			+		+							

TABLE 18.—Tumor cells in the blood and incidence of lung metastases in noninbred Japanese rats intraperitoneally inoculated with 17 strains of rat ascites hepatomas

Tumor cells in blood	Number of rats examined	Number with lung metastases	Percent with lung metastases
Present	38	28	74
Absent	332	37	11

## METASTASIS AND CHEMOTHERAPY

Surgery, radiation, and chemotherapy are commonly used for the clinical treatment of cancer metastasis, but there are many limitations and much difficulty in their application to patients. Surgery and radiation cannot be used when multiple metastases have occurred. In such conditions,

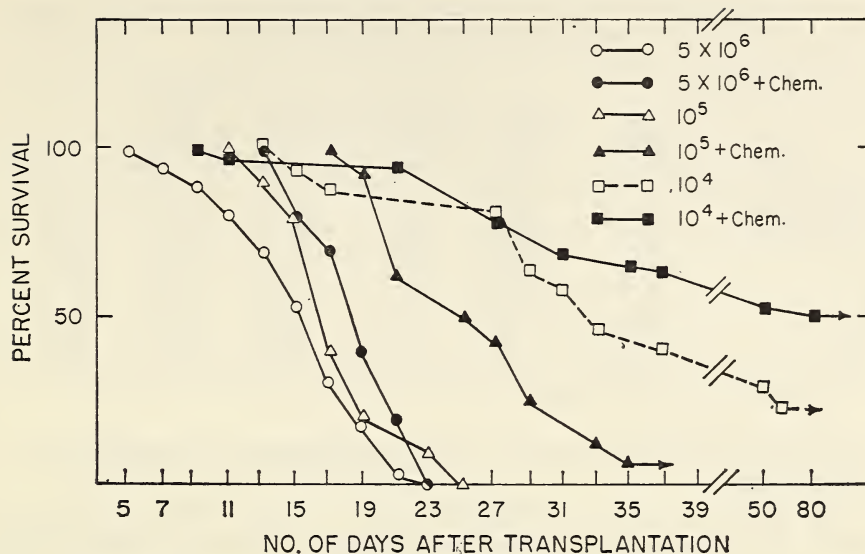


TEXT-FIGURE 1.—Effect of Nitromin (nitrogen mustard *N*-oxide) on Yoshida sarcoma in rats.

chemotherapy is used as a last resort, but usually fails to give satisfactory results. If we consider that the development of metastasis should be inhibited at a premetastatic stage, in which only small numbers of tumor cells exist as seeds of metastasis, the role of chemotherapy should be greater than that of surgery or radiation. Those cells are not targets that can be reached by surgery or radiation when they are disseminating in the body through the blood stream, spreading into the lymphatics, moving in the body cavity, or latent in the tissues.

In tests of chemotherapeutic substances on the transplantable tumors, it has been generally found that the earlier treatment is given the better are the results. An example is shown in text-figure 1. Nitromin was administered at various stages after transplantation. A high survival rate and prolongation of life were marked when the administration was begun early (4 days) or immediately after transplantation, but the animals succumbed earlier when administration was begun later (7 days after transplantation). The better results obtained in the earlier period suggest that the growth of the tumor cells was not yet advanced and that there were few target cells for chemotherapy. This seems to be supported by the results in other experiments (text-fig. 2). Tumor cells of MH 134 ( $5 \times 10^6$ ,  $1 \times 10^5$ , and  $1 \times 10^4$ ) were transplanted into the peritoneal cavity of C3H mice. Chromomycin A<sub>3</sub> ( $4 \mu\text{g}/\text{mouse}$ ) given 1 day after transplantation prolonged life, and there was a high survival rate in the mice in which a small number of cells was transplanted.

Many factors determine the effectiveness of a chemotherapeutic substance: The cancer cells must be sensitive to it, sufficient substance must



TEXT-FIGURE 2.—Growth-inhibiting effect of chromomycin A on intraperitoneally inoculated cells of tumor MH 134.

reach the cells, and the number of cells must be considered. A better effect can be expected when the number of cells is small. In other words, chemotherapy is most effective in cases in which the primary tumors are removed surgically and only a small number of cells, which might be the source of future recurrence and metastasis, remain in the body. This concept is supported by the experimental results described in table 9.

#### FACTORS REGULATING THE GROWTH OF SMALL NUMBERS OF TUMOR CELLS

When the mouse ascites hepatomas were transplanted intravenously, all animals died from marked tumor growth in the lungs. The question arises whether all of the transplanted cells could survive and proliferate at the sites in the lungs reached through the blood stream. Therefore, mice inoculated with about  $10^6$  MH 134 cells in the caudal vein were killed at various intervals after inoculation to observe the number of tumor cells in the lung tissue. The number per  $\text{mm}^2$  in the lungs decreased gradually, and the proportion of degenerating cells increased and was almost 60 percent of all remaining tumor cells 3 days after inoculation (table 19). At about this time, the tumor cells were located in the capillaries without showing any extravascular extension; in the later period, the initial growth extended into the interstitial tissue surrounding the blood vessels of the lungs. The results indicate that most of the embolic tumor cells perished in the sites of arrest but that the growth of tumor

TABLE 19.—Number of embolic tumor cells in the lungs of mice inoculated intraperitoneally with MH 134 tumor cells

Time	Lung area* (mm <sup>2</sup> )	Number of tumor cells	Number of degenerating cells	Number of tumor cells per mm <sup>2</sup> (A)	Number of degenerating cells per mm <sup>2</sup> (B)	B/A × 100
Immediately	34.1	1,625	136	47.64	3.99	8.4
0.5 hours	48.2	1,430	134	29.67	2.78	9.4
3	54.1	1,864	201	34.11	3.72	10.9
6	61.4	365	117	6.13	1.97	32.1
12	79.6	178	81	2.78	1.23	45.1
24	54.1	94	44	1.76	0.80	46.6
48	57.7	52	31	0.93	0.57	59.6
72	61.6	17	10	0.23	0.13	58.8

\*Total of the lung areas measured in 3 mice.

occurred from the remaining few cells and developed to cause the death of the hosts.

This gradual decrease and degeneration of tumor cells in the lung tissue were demonstrated through transplantation of lung tissues, of the same donors as in the previous experiment, into the peritoneal cavity of the recipients.

The decrease in transplantability and the prolonged latent period before the appearance of tumor cells in the ascites indicated that the number of the remaining tumor cells might be very small (table 20).

TABLE 20.—Detection of intravenously inoculated MH 134 cells in the lungs by intraperitoneal inoculation into recipient mice of minced lung tissue from donor mice

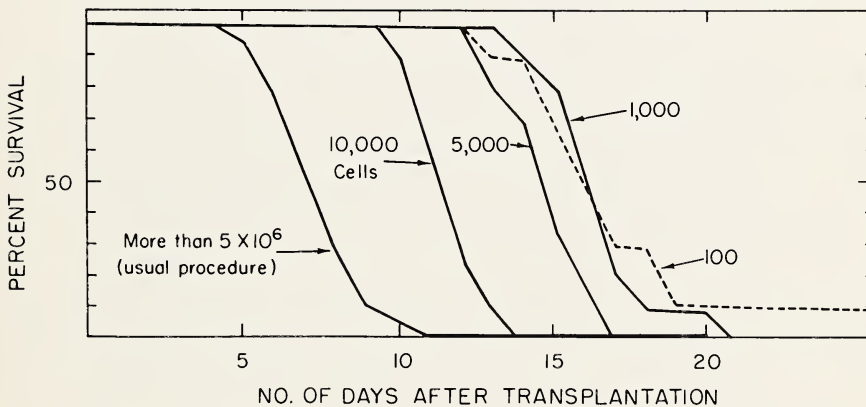
Time	Positive* detection	Time for appearance of tumor cells (days)†		
		Earliest	Last	Average
Immediately	8/8 (100)	3	7	6.8
0.5 hours	5/5 (100)	3	10	6.8
3	8/10 (80)	3	15	9.5
6	6/7 (86)	10	29	18.3
12	5/5 (100)	14	48	26.4
24	4/7 (57)	10	34	18.5
48	3/7 (43)	20	54	36
72	3/6 (50)	10	22	17.3
5 days	4/7 (57)	6	23	13.5
10	4/6 (67)	3	20	8
15	6/6 (100)	3	23	8.1

\* The tissues of the lungs of the donors were inoculated into the peritoneal cavity of the recipients. Figures in parentheses indicate percent.

† The earliest, last, and average time until the tumor cells were detected first in the ascites of the recipients.

In spite of the high transplantability of these tumors, not all the transplanted cells survived and proliferated in the sites in which they were placed. It appears that the hosts do not surrender willingly to the growing tumor cells, but first show a marked reaction. Successful trans-

plantation seems to be effected when the tumor growth overcomes the host reaction. These phenomena can be observed not only in intravenous transplantation but also in intraperitoneal, subcutaneous, and intratracheal transplantations. It is reasonable to consider that the living body possesses the power to react to the invasion of tumor cells. If a way can be found to strengthen the resistance of the hosts to tumor growth, there may be an approach to the final objective—the cure of cancer. Some preliminary results were obtained in a series of experiments with “Donryu” rats, known to be susceptible to the growth of Yoshida sarcoma, and Yoshida sarcoma cells. When the tumor cells are transplanted their growth is quite uniform, and almost 100 percent of the animals die from the tumors. Even when small numbers of tumor cells are transplanted, the animals die from the tumors, though they survive longer (text-fig. 3). When mouse



TEXT-FIGURE 3.—Transplantability of Yoshida sarcoma in “Donryu” rats (a highly susceptible strain).

tumor cells were transplanted into the peritoneal cavities of Donryu rats, the tumor cells first proliferated and then stopped growing within 7 to 10 days after transplantation. After the tumor cells had disappeared from the ascites, most of the animals survived. These animals were given transplantations of Yoshida sarcoma cells such as  $1 \times 10^6$ ,  $1 \times 10^5$ ,  $1 \times 10^4$ , or  $1 \times 10^3$ . When many cells were used, the animals died from the tumors but when few cells were used many survived and the cells disappeared after temporary growth. In the animals surviving these procedures, tumor growth was inhibited even when a large number of cells was used (table 21). This might be interpreted to indicate that the host condition, in which a small number of Yoshida sarcoma cells could grow, was changed to one in which the growth was regulated by pretreatment, such as heterotransplantation. Various kinds of treatments effective in inhibiting the growth of a small number of tumor cells are being investigated, and the results will be reported elsewhere.

TABLE 21.—Development of resistance to growth of Yoshida sarcoma transplanted into "Donryu" rats

Transplants	Number of cells transplanted					10 <sup>7</sup> or more
	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>7</sup>	
Heterograft (mouse tumors)	—	—	—	39/40 (97%)	83/85 (98%)	97/220 (44%)
Homograft I	27/31 (87%)	22/33 (67%)	22/46 (48%)	0/20 (0%)	—	—
Homograft II	—	—	—	25/33 (76%)	16/16 (100%)	—
Homograft III	—	—	—	—	24/24 (100%)	—

## SUMMARY

To study the mechanism of metastatic formation, transplantable animal tumors, especially ascites tumors, were investigated for their metastasizing ability. Of these, the MH 134, MH 129F, and MH 129P mouse ascites hepatomas had a marked ability to form metastases, *e.g.*, recurrence and metastasis following surgical interventions could be obtained in the experimental procedures. In addition, selective lymph node metastases could be designed experimentally by transplanting those tumor cells into the tails of animals.

From the steps involved in the development of a metastatic lesion, it is suggested that small numbers of cells might be the seeds of metastasis. Thus, the appearance of tumor cells in the circulating blood was investigated in the tumor-bearing animals, and the correlation between presence of tumor cells in the blood and metastatic formation was discussed. It is believed that the appearance of tumor cells in the blood is, at least, an indicator of metastatic development.

The inhibiting effects of chemotherapy were evaluated, especially on the growth of small numbers of tumor cells and, thus, chemotherapy was suggested as a factor in the prevention of metastatic development.

Factors which might control the growth of tumor cells should be investigated, especially if the number of cells is very small, because the resistance of the hosts might be correlated with the development of metastasis and also with the viability of tumor cells.

## REFERENCES

- (1) YOSHIDA, T.: Metastasis and chemotherapy. *Acta Un Int Cancer* 16: 496-499, 1960.
- (2) SATO, H.: Experimental studies on the mechanism of metastasis formation. *Acta Path Jap Suppl*: 685-706, 1959.
- (3) SATO, H., BELKIN, M., and ESSNER, E.: Experiments on an ascites hepatoma. III. The conversion of mouse hepatomas into the ascites form. *J Nat Cancer Inst* 17: 1-21, 1956.

- (4) KAWASHIMA, Y.: Studies on metastasis of cancer. V. Recurrence and metastasis formation following operation of tumors of C3H mouse ascites hepatomas. *Fukushima J Med Sci* 10: 433-446, 1960 (written in Japanese).
- (5) ODASHIMA, S.: Personal communication based on the unpublished data, 1961.
- (6) SAITO, T., KAWASHIMA, Y., and SATO, H.: Transplantable strains of breast cancer in C3H/HeN mice. *Gann* 49: (Suppl) 189, 1958.
- (7) DUNHAM, L. J., and STEWART, H. L.: A survey of transplantable and transmissible animal tumors. *J Nat Cancer Inst* 13: 1299-1377, 1953.
- (8) STEWART, H. L. *et al.*: Transplantable and Transmissible Tumors of Animals. Atlas of Tumor Pathology, Fascicle 40, Section XII, Washington, D.C., Armed Forces Institute of Pathology, 1959.
- (9) KETCHAM, A. S., KINSEY, D. L., WEXLER, H., and MANTEL, N.: The development of spontaneous metastases after the removal of a "primary" tumor. *Cancer* 14: 875-882, 1961.
- (10) SATO, H., KAWASHIMA, Y., KANNO, K., MUNAKATA, H., and SAITO, T.: Studies on the transplantability of the mouse ascites hepatomas. *Fukushima J Med Sci* 5: 155-173 1958.
- (11) SATO, H.: Experimental studies on the role of cancer chemotherapy for prevention of recurrence and metastasis formation in malignant tumors. *Acta Un Int Cancer* 16: 763-768, 1960.
- (12) KAWASHIMA, Y.: Studies on metastasis of cancer. VI. Effect of Nitrogen mustard N-oxide (Nitromin) upon post-operative recurrence and metastasis formation. *Fukushima J Med Sci* 10: 447-454, 1960 (written in Japanese).
- (13) KOCH, FR. E.: Zur Frage der Metastasenbildung bei Impftumoren. *Zeitschrift für Krebsforschung* 48: 495, 1939.
- (14) KANNO, K.: Studies on metastasis of cancer. VII. Experiments on lymphatic spread of tumors. *Fukushima J Med Sci* 10: 473-488, 1960 (written in Japanese).
- (15) SATO, H.: Studies on the role of cancer chemotherapy for prevention of lymph node metastasis. *Cancer Chemotherapy Reports* 13: 33-40, 1961.
- (16) SAITO, T.: Experimental studies of ascitic conversion of mammary cancer of C3H mice. Establishment of an ascites tumor FM3A. *Fukushima J Med Sci* 12: 335-352, 1962 (written in Japanese).
- (17) SATO, H.: Cancer cells in the circulating blood with reference to cancer metastasis. WHO Public Health Pap 26: 675-681, 1962.
- (18) MUNAKATA, H.: Studies on metastasis of cancer. XI. Hematogenous dissemination of tumor cells of the mouse ascites hepatoma (MH134). *Fukushima J Med Sci* 11: 1277-1280, 1961 (written in Japanese).
- (19) ———: Studies on metastasis of cancer. X. Hematogenous dissemination of tumor cells of the rat ascites hepatoma. *Fukushima J Med Sci* 11: 1263-1276, 1961 (written in Japanese).
- (20) ———: Studies on metastasis of cancer. XII. Experiments on the outcomes of tumor cells in the blood. *Fukushima J Med Sci* 11: 1281-1286, 1961 (written in Japanese).

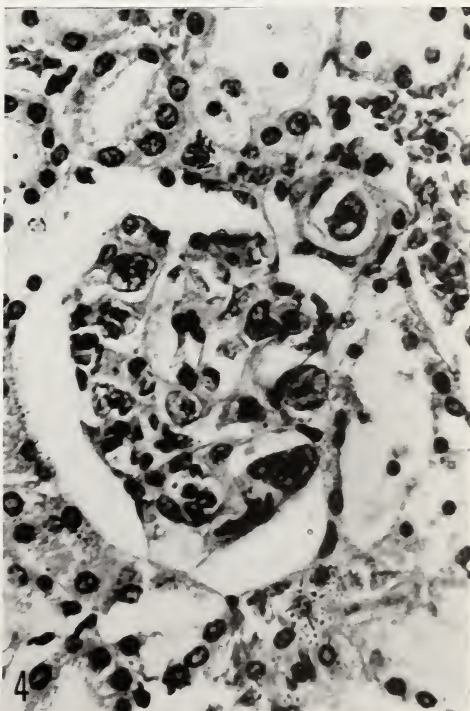
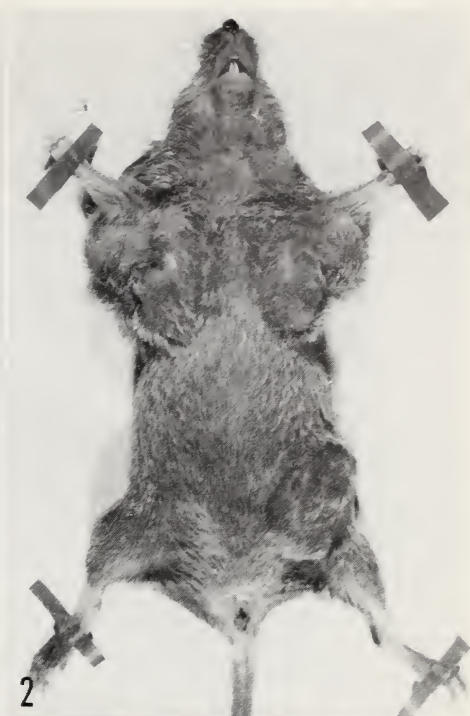
## PLATE 48

FIGURE 1.—Solid tumor grown in the back of a C3H mouse that died 30 days after subcutaneous inoculation of MH 134 ascites tumor cells.

FIGURE 2.—Marked metastasis in the lymph nodes of a C3H mouse in which subcutaneously inoculated MH 134 tumor was surgically removed 2 weeks after inoculation. Mouse survived 80 days.

FIGURE 3.—Metastasis in the lung of a C3H mouse in which subcutaneously inoculated MH 129F tumor was surgically removed 2 weeks after inoculation. Mouse survived 49 days.

FIGURE 4.—Metastasis in the kidney, showing tumor cells embolizing in the capillaries of the glomerulus. MH 134 tumor was transplanted subcutaneously and was removed 2 weeks after inoculation. Mouse survived 42 days.



## PLATE 49

FIGURE 5.—Marked tumor growth in the tail of C3H mouse 20 days after inoculation of MH 134 tumor.

FIGURE 6.—Recurrence of tumor at the amputated end of the tail and metastasis in the retroperitoneal and superficial (inguinal, axillary) lymph nodes. The tail was amputated 10 days after inoculation of MH 134 tumor. Mouse survived 29 days.

FIGURE 7.—Metastasis in the sciatic lymph nodes of a mouse in which the tumor-bearing tail was amputated 3 days after inoculation. Inguinal and popliteal lymph nodes are also enlarged with metastasis. Mouse survived 42 days.

FIGURE 8.—Marked metastasis in the retroperitoneal region including lumbar and renal lymph nodes. The tumor-bearing tail was amputated 3 days after inoculation. Mouse survived 28 days.





## Yoshida Sarcoma as a Tool for Cancer Chemotherapy Screening<sup>1</sup>

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SINCE Yoshida's first report in 1949 (1, 2) on the cancer chemotherapy screening method with Yoshida sarcoma, much work has been done in Japan, and numerous synthetic compounds, antibiotics, and plant extracts have been tested by this method. Nitromin (3), sarkomycin (4), carzinophilin (5), mitomycin (6), and chromomycin (7, 8) are examples of chemotherapeutic compounds tested with Yoshida sarcoma. In the present paper no attempt will be made to list the numerous compounds tested on each tumor or to discuss the problems using spontaneous versus transplantable tumor or of solid versus ascites tumor as screening tools. We have used the Yoshida sarcoma as a routine method since 1953 and the important findings will be discussed.

### ESTABLISHMENT OF YOSHIDA SARCOMA AS A ROUTINE SCREENING METHOD

In 1947, at a meeting of the Pathological Society of Japan, Yoshida reported on the general use of the Yoshida ascites sarcoma. It was apparent that this tumor might be useful not only for fundamental research on cancer chemotherapy but also that it might be more valuable to use than solid tumors, because the tumor cells could be examined individually, both before and after the administration of chemotherapeutic agents. In 1950, he published the following systematized screening test (2).

- A. 1) The maximum tolerated dose (MTD) was determined.
- 2) A near MTD was given intraperitoneally to rats bearing a 3- to 4-day-old Yoshida sarcoma.
- 3) The ascitic fluid was examined at  $\frac{1}{2}$ , 1, 3, 6, 12, and 24 hours after the injection.

In fresh material:

- (a) The number of tumor cells in 1 cm<sup>3</sup> of ascitic fluid was counted—the decrease in the number of cells after treatment and the recovery rate in 24 hours were determined.

<sup>1</sup> We appreciate the consistent help of Messrs. T. Komeda, K. Otsu, T. Kimura, I. Miura, T. Morita, Misses Y. Nishioka, M. Arakawa, and K. Osaka, and also of the Chemical Division of the Laboratories and the Division of Animal Supply.

In Giemsa-stained smears:

- (a) The cellular changes produced by the treatment were evaluated in the nucleus, cytoplasm, and chromosomes.
  - (b) The progression and regression of the changes were considered, and the damage to the total tumor cells was estimated.
  - (c) Cellular reaction: After any substance is injected, an acute inflammatory infiltrate consisting of neutrophils, eosinophils, and monocytes is observed in the tumor ascites. The degree of this reaction differs markedly with various agents, and the number of reactive cells and the rapidity of their appearance and disappearance must be evaluated. These observations may also serve as an indication of the toxic effect of the agent on normal tissue cells. If the tumor cells are intensively damaged but the reactive cells are numerous and do not show any evident degenerative changes, the agent tested is regarded as suitable.
- 4) After 24 hours if the animal has survived, the ascitic fluid is examined once a day, until the animal dies, to determine if a single dose prolongs the lifespan. An autopsy examination is performed to determine the cause of death and the extent of tumor involvement.
- B. 1) Chemotherapeutic substances passing screening A are examined further to ascertain their ability to produce a "cure" or to prolong life. Repeated administration of a half lethal dose or minimum effective dose is given at suitable intervals.
- 2) When a substance is definitely effective after intraperitoneal injection, it is tested further by the subcutaneous route of administration

When this screening test was routinely used in our laboratory in 1953, minor modifications were necessary: The approximate MTD was determined in mice instead of rats, and tumor ascites at 3, 6, 24, and 48 hours were examined in Giemsa-stained smears. In order to train technical assistants to screen the slides for information on the effectiveness of the agent, a training course was given similar to the type given for checking vaginal smears by the Papanicolaou method (9). A junior assistant first checks and files the protocol. Then a senior assistant checks without looking at the filed protocol and the findings are compared. A pathologist makes a final check and discusses his finding with his assistants. Each again checks the slide before a final decision is made.

In this screening, the criteria involve the effectiveness of a compound at a cellular level and the prolongation of life of the experimental animals. Various requisites, such as "quality control" of Cancer Chemotherapy National Service Center protocol, are also considered (10), but in the present paper only the two criteria will be discussed.

## CYTOLOGICAL CHANGES OF YOSHIDA SARCOMA CELLS AFTER ADMINISTRATION OF CHEMOTHERAPEUTIC AGENTS

Cytological changes produced after the administration of drugs are important indicators in this screening procedure. The following six are applied to the over-all decision of the effects (11): 1) cytological changes in tumor cells not observed in controls; 2) frequent changes seldom observed in controls; 3) marked decrease or increase in the frequency of

mitotic figures, including arrest of mitosis and mitotic aberrations; 4) marked decrease in number of tumor cells, such as marked cytolysis; 5) continuation of these changes for a considerable time; 6) apparent dose-response relationship of the changes—the higher the dose, the more noticeable the changes.

When the cytological changes were evaluated as "effective," they were carefully compared with those of known chemotherapeutic agents whose effects had already been extensively studied.

### Nitromin Effect

The characteristic changes by alkylating agents have been designated as Nitromin effect, because of the pronounced changes produced by the drug when Yoshida sarcoma screening procedures were first used. As reported elsewhere (3), the effects consist of a gradual decrease in the frequency of mitotic cells and the late appearance (12–24 hours after the injection) of abnormal mitosis, such as bridge formation, lagging, and coagulation of chromosomes, leading to giant, multinucleated cells and degeneration of tumor cells. These peculiar sequences of cytological changes corresponded with the dose-response relationship of Nitromin and to the survival time of tumor-bearing rats. Hundreds of derivatives have been synthesized and checked by this criteria.

But other alkylating agents do not show the same effects, *e.g.*, Busulfan (12) and Endoxan (13) widely used in chemotherapy show different changes. On the other hand, compounds besides the alkylating agents also show the Nitromin effects, such as carzinophilin (14) and mitomycin (15). These two antibiotics not only show the typical Nitromin effects but also destroy the nucleus and cytoplasm soon after the drug is administered. Whether these cytological changes occur because of the chemical structure (recently established) of mitomycin-aziridinyl, quinone-urethan groups or whether the chemical structures causing the "Nitromin" effect are limited to so-called alkylating groups (16)—such as dichlorethyl, ethylenimine, methanesulfonate, and epoxide—will be explored in the future. The relation between morphological peculiarity and biochemical mechanism of the predominantly DNA-attacking agents presents challenging problems.

### Colchicine Effect

At the beginning of the present research we found colchicine to be an interesting compound. Its characteristic features are arresting of metaphase, a high frequency of metaphase beginning relatively early (3–6 hours after administration), and star and scattered metaphase, usually followed by multinucleated giant cells (17, 18).

The derivatives of colchicine were tested (19) when the chemistry of substance F was studied by a group of our colleagues (Y. Ueno and col-

laborators). The activity of the derivatives on the Yoshida sarcoma cells is shown in tables 1 and 2. Some of the following changes were noted:

1) When  $R_1$  or  $R_2$  was changed but  $R_3$  was not (as in colchicine), the toxicity was lower, but the activity was also decreased in all the compounds except Cl-3.

2) When  $R_3$  was replaced by an OH group it had no activity, but when  $R_3$  was substituted for an  $NH_2$  or  $NHCH_3$  group, the LD50/MED value increased, *e.g.*, *N*-methyl-colchiceineamide (Cl-7). In the demecolceineamides, Cl-9 with a monomethylamino group was more active than Cl-3 when their LD50/MED value was compared.

3) The *N*-nitroso compound showed a high LD50/MED value. However, since it is sparingly soluble in water, it was tested as a suspension and the value obtained must be considered with reservation.

4) At decreasing concentrations the activity of each compound was checked by a metaphase count. At the lowest dose the maximal metaphase accumulation occurred in the 2d hour after injection, and at the highest dose 6 hours or later. The accumulation was most frequent in Cl-4, Cl-7, Cl-8, and Cl-9.

5) When the specimens were examined at 6 hours, star metaphase was more frequent when the compounds (Cl-7, Cl-8, and Cl-9) contained an  $NH_2$  or  $NHCH_3$  group on the  $R_3$  ring than an  $OCH_3$  group (Cl-1, Cl-2, and Cl-4).

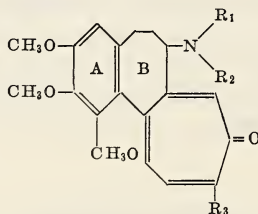
TABLE 1.—Derivatives of colchicine tested

Cl-1	Colchicine
Cl-2	<i>N</i> -Methyl-deacetyl-colchicine*
Cl-3	<i>N</i> -Nitroso- <i>N</i> -methyl-deacetyl-colchicine†
Cl-4	<i>N</i> -Methyl-colchicine‡
Cl-5	<i>N</i> -Methyl-colchiceine
Cl-6	<i>N</i> -Methyl-deacetyl-colchiceine
Cl-7	<i>N</i> -Methyl-colchiceineamide
Cl-8	<i>N</i> -Methyl-deacetyl-colchiceineamide
Cl-9	<i>N</i> -Methyl-deacetyl-colchiceine-methyl-amide

\*Demecolceine

†*N*-Nitroso-demecolceine

‡*N*-Acetyl-demecolceine



Number	$R_1$	$R_2$	$R_3$
Cl-1	H	$COCH_3$	$OCH_3$
Cl-2	H	$CH_3$	$OCH_3$
Cl-3	NO	$CH_3$	$OCH_3$
Cl-4	$CH_3$	$COCH_3$	$OCH_3$
Cl-5	$CH_3$	$COCH_3$	OH
Cl-6	$CH_3$	H	OH
Cl-7	$CH_3$	$COCH_3$	$NH_2$
Cl-8	H	$CH_3$	$NH_2$
Cl-9	H	$CH_3$	$NHCH_3$

TABLE 2.—Minimum effective dose (MED)\* and LD50 of the derivatives of colchicine

Number	LD50 mg/kg (mouse, intraperi- toneally)	MED $\mu$ g/kg	LD50/MED
Cl-1	1.0	12.3(0.3)†	81.3
Cl-2	8.0	98.7(2.7)	81.1
Cl-3	11.3	15.3(0.4)	719.6
Cl-4	4.3	159.3(3.9)	30.0
Cl-5	400.0	—	—
Cl-6	400.0	44444.4(1324.3)	9.0
Cl-7	35.0	47.4(1.1)	738.4
Cl-8	67.0	827.2(21.3)	81.0
Cl-9	53.0	218.1(5.9)	243.0

\*Minimum dose which caused the cytological effects on the Yoshida sarcoma cells.

†Mol  $\times 10^{-7}$  in parentheses.Plant Extracts From *Vinca Rosea*

Although colchicine and/or its derivatives are not as frequently used in medical practice as cancer chemotherapeutic compounds, the Yoshida sarcoma was very sensitive to the colchicine derivatives. In 1959, during the course of screening many materials, a very interesting plant extract, *Vinca rosea major*, was checked in a 50 percent methanol extract by the characteristic features of metaphase arrest. After examining the biological activity of the 50 percent methanol extract from various parts of *Vinca rosea major* and other related plants, a chemical approach to isolation and purification procedures of the active principles was started, with the cytological method as a guide. Soon the fraction designated as "S-6" was obtained and the metaphase arrest was 100  $\mu$ g per kg. Dr. M. Goto and Dr. S. Imai obtained "CE-II, III" in crystalline form; the minimal arrest of mitosis was 1  $\mu$ g per kg.

The results of a comparative study on the metaphase arresting activity of fraction S-6 and Vincalukoblastine<sup>2</sup> (VLB) (20,21) are shown in table 3. The mitotic arresting activity of VLB on the Yoshida sarcoma cell is indicated in table 4. VLB is slightly more active in an alcoholic solution than in a saline solution. Yoshida sarcoma was more sensitive to VLB than Ehrlich ascites carcinoma (22) (table 5).

TABLE 3.—Comparison of the metaphase arresting activity of Vincalukoblastine and S-6 fraction on the Yoshida sarcoma cells and their leukopenic effect in rats

	Minimum dose for metaphase arrest* ( $\mu$ g/kg)	Minimum dose for leukopenic effect† ( $\mu$ g/kg)
S-6	60	10
Vincalukoblastine	7-15	1
Ratio	$\frac{1}{10}$ - $\frac{1}{4}$	$\frac{1}{10}$

\*Indicated by the appearance of star or scatter metaphase after 6 hours of administration.

†Indicated by 50 percent decrease in total leukocyte count.

<sup>2</sup> Kindly supplied by Dr. Johnson.

TABLE 4.—The comparison of the cytological effects of Yoshida ascites sarcoma cells by single intraperitoneal administration of Vincalukoblastine\*

Dose ( $\mu$ g/kg)	Hours after injection :							
	1		3		6		24	
	Saline	Ethanol	Saline	Ethanol	Saline	Ethanol	Saline	Ethanol
1000	A	A	A	A	A	A	C	C
125	A	A	A	A	A	A	C	C
8	B	A	B	B	B	B	—	C
2	B	B	B	B	B	B	—	—
0.5	B	B	—	B	—	B	—	—
0.1	B	B	—	—	—	—	—	—
0.08	—	—	—	—	—	—	—	—
							Survival of rats (days)	
							Saline	Ethanol
							17	14 cured, 14
							10 11	13 14
							8 8	9 9
							8 10 (cured)	11
							9 9	9 17
							9 9	7 9
							8 9	9 10

\*A = scattered ball metaphase; B = star metaphase; C = multinucleated giant cell formation.

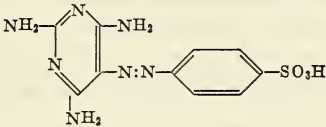
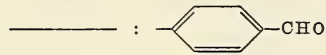

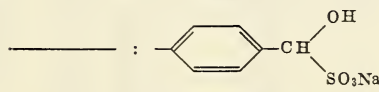
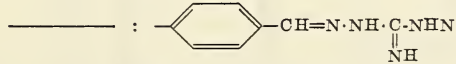
TABLE 5.—Comparative effects of Vincal leukoblastine on various ascites tumors

Dose (mg/kg)	Ascites tumor	Arrested metaphase		
		Hours after injection	Mitotic index	Percent metaphase
1.0	Ehrlich	12-24	8.0-9.9	72-80
	L1210	12	12.7	80.8
	Yoshida	9-12	59.6-63.6	100
0.5	Ehrlich	72	28.6	82.4
	Yoshida	9-12	59.6-83.4	100

## Derivatives of 5'-Phenylazopyrimidine

Derivatives of pyrimidine were used to synthesize potential anti-metabolites. A group of derivatives of 2,4,6-triamino-5-phenylazopyrimidine was selected and tested by a lactobacillus inhibition system and cytologically with Yoshida sarcoma cells and Ca-755 solid tumor system (23). Para position of the phenyl moiety was examined carefully, and Py-64 and Py-80 were selected for their biological effects. Further chemical modifications were tried, and when we reached Py-114, specific cytological characteristics such as abnormality of chromosomes were found. Finally, we synthesized Py-122 which caused characteristic colchicine-like mitotic arrest. The chemical structures of these derivatives are shown in table 6.

TABLE 6.—Chemical structure of derivatives of 5'-phenylazopyrimidine tested

Py-64	
Py-114	
Py-115	
Py-116	
Py-122	

Because Py-122, 4-(2,4,6-triamino-5-pyrimidinylazo)benzaldehyde guanyldiazotone, was not soluble in water, Dr. H. Mima in the physico-chemistry division collaborated with us in an effort to find a solvent. It was found that Py-122 was soluble in a 0.5 percent glutamic acid solution or 1.5 percent gluconic acid solution, and the final compound Py-122-GLT was readily soluble in water. Cytological studies of the effects of Py-122

glutamate on the dividing cells of the Yoshida sarcoma and those of the ascites hepatomas were reported elsewhere (24). In accordance with the increase in solubility, the minimum effective dose, measured by the arrest of mitosis, decreased enormously, although the LD50 values were slightly decreased (table 7).

TABLE 7.—The difference in the activity of Py-122 in various solvents

	Solvent or suspending medium	Toxicity, mg/kg (mouse, intraperitoneally)	MED (mg/kg)
Py-122 (CMC)	0.2% Carboxymethyl-cellulose	Calculated 1,000	50
Py-122 (G)	Gum arabic		10
Py-122 (HS)	10% Horse serum	Calculated 100	1
Py-122 (GLT)	0.5% Glutamic acid	Intravenously 54, intraperitoneally, 268	0.05
Py-122 (GLC)	1.5% Gluconic acid or gluconolactone	Intravenously 60, intraperitoneally, 340	0.01

It is not known whether the 100-fold, or more, increase in activity (expressed as a decrease in MED dose) is dependent on the difference in the physical state (solubility) of the compound or if there are other factors, such as the increase in permeability of cell membrane, associated with these phenomena. A compound, guanyldiazotization product, *p*-acetylamino benzaldehyde, Py-122a, with no pyrimidine moiety was tested on the Yoshida sarcoma cells and found to be slightly active, thus indicating that guanyldiazotization itself was involved in metaphase arrest. This group of moiety is quite similar to that found in methyl GAG (25).

Py-122 and its derivatives have been tested in various other screening systems at our laboratory as well as at the Sloan-Kettering Institute (table 8). Py-64 and Py-80 have been tested clinically and when given in

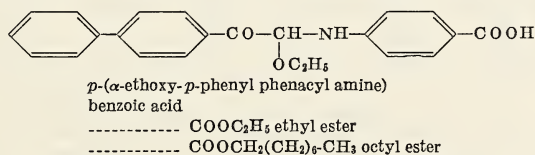
TABLE 8.—Antitumor activity of Py-122

Tumor	Dose mg/kg (7-14 days intraperitoneal injections)	T/C % (2 weeks, size)	T/C % (2 weeks, weight)	Remarks
Yoshida sarcoma	1	70	38	Donryu rat
	10	69	88	
	20	70	42	
Adenocarcinoma 755	1	86		C57BL
	10	66		
	20	61		
Mouse ascites hepatoma 134	1	91		C3H
	10	103		
	20	127		
Mouse leukemia 36	1	133		CF1
	10	97		
	20	125		
Sarcoma 180 Crocker	1	82		CF1
	10	80		
	20	56		
Ehrlich (solid)	1	71		CF1
	10	77		
	20	171		

combination with 6-mercaptopurine and prednisolone to patients with chronic myelocytic leukemia seemed more effective than 6-mercaptopurine and prednisolone alone (26).

### Other Compounds Screened for Colchicine-Like Activity

New antibiotics, new plant extracts, and various synthetic compounds are included, such as: (a) a group of vitamin derivatives which contain -S-S- linkage, (b) xenalamine and its derivatives (27), (c) some heterocyclic compounds, and (d) several completely new types of compounds that were selected for their dehydrogenase-inhibiting activity of Ehrlich ascites tumor cells using a cell agar plate method. Attention was focused on the cytological effects of xenalamine because of the report on its inhibitory effect upon multiplication of influenza virus (28). The structure of the compound is shown. Its ethyl and octyl ester were compared. The



effect was greatly increased when the soluble ethyl ester was used rather than the insoluble free acid (tables 9 and 10); the octyl ester possessed no activity in arresting metaphase of Yoshida sarcoma cells. Comparison of the effect of several related compounds and several components of the compound were also tested (table 11). The results showed that the activity may depend on a "guanyl hydrazone" moiety. Results of other chemicals (a), (c), and (d) will be reported later.

TABLE 9.—The effect of  $p$ -( $\alpha$ -ethoxy- $p$ -phenyl phenacyl amine) benzoic acid on the mitotic index of Yoshida ascites sarcoma cells *in vivo*

Dose (mg/kg)	Duration (hr)	Mean No. of cells per 10 <sup>3</sup> scored					Mitotic index
		Inter- phase	Pro- phase	Meta- phase	Post meta- phase	Percent meta- phase (total mitotic cells)	
100	0	971	10	14	5	48	2.9
	3	964	3	27	6	75	3.6
	6	971	10	7	12	24	2.9
	24	982	3	9	6	50	1.8
	48	983	7	7	3	41	1.7
250	0	975	5	16	4	64	2.5
	3	899	7	90	4	89	10.1
	6	930	3	65	2	93	7.1
	24	989	4	2	5	18	1.1
	48	983	1	12	4	70	1.7
500	0	968	5	18	9	56	3.2
	3	960	5	35	0	88	4.0
	6	916	3	79	2	94	8.4
	24	991	2	5	2	56	0.9
	48	986	2	9	3	64	1.4

TABLE 10.—Effect of *p*-( $\alpha$ -ethoxy-*p*-phenyl phenacyl amine) benzoic acid ethyl ester on the mitotic index of the Yoshida ascites sarcoma cells *in vivo*

Dose (mg/kg)	Duration (hrs)	Mean No. of cells per 10 <sup>3</sup> scored					Mitotic index
		Inter- phase	Pro- phase	Meta- phase	Post meta- phase	Percent metaphase (total mitotic cells)	
10	0	978	4	12	6	50	2. 2
	6	904	8	69	9	72	9. 6
	24	974	6	9	11	35	2. 6
	48	988	3	4	5	33	1. 2
50	0	984	3	10	3	63	1. 6
	6	724	6	276	8	95	27. 6
	24	967	6	17	10	52	3. 3
	48	966	5	22	7	65	
100	0	977	9	13	11	57	2. 3
	6	628	10	362	0	97	37. 2
	24	960	9	19	12	50	4. 0
	48	995	8	7	10	28	2. 5

TABLE 11.—Effect of related compounds tested

Compound	Dose (mg/kg)	C-Metaphase effect (6 hrs)
Xenalamine	100	—
	250	±
	500	±
Xenalamine ethyl ester	10	±
	50	+
	100	++
Xenalamine octyl ester	10	—
	50	—
	100	—
4-Biphenyl glyoxal thiosemicarbazone	10	—
	100	—
	500	—
4-Biphenyl glyoxal guanylhdyrazone	10	+
	100	+
	500	+
Biphenyl	100	—
<i>p</i> -Aminobenzoic acid	100	—

More experimental studies must be done before these compounds can be classified as cancer chemotherapeutic agents. Several new derivatives of known compounds belonging to this category, such as podophyllin-säureaethylhydrazid, benzylidenierte glucoside, from podophyllum emodi (29) and Vincristine (30), have become available.

#### Other Cytological Effects

Various changes are observed after the administration of different compounds and the destructive changes of the cell and nuclei are the most

frequent. We have tentatively divided the most frequently observed changes into the following two categories, keeping cellular changes which occur in the heterotransplantation in mind: (a) immediate destruction of tumor cells and (b) late and continuing destruction of tumor cells.

Both changes are altered by: (a) the response of the host, expressed by the appearance of reactive cells and exudate, accompanied simultaneously with the destruction of tumor cells and (b) those appearing after the destructive changes of tumor cells have occurred. Generally speaking, the immediate cellular change accompanying the host's reaction has not been regarded as effective, even though the minimal tumor-cell-destruction dose is very small, because such compounds are often toxic and the host response always occurs simultaneously. But when the cellular changes appear late the results often may be promising.

Four derivatives of Sb preparations are reported (31) to show marked pyknosis and karyorrhexis, with a continued decrease in tumor cells and no abnormality in chromosomes. Reactive neutrophils and monocytes are abundant and persist, resembling the findings observed in spontaneous regression of tumors.

Emetine hydrochloride (32) was used because of the effects of the Sb preparation as one of the amoebicidal chemotherapeutic agents, demonstrated by karyorrhexis, karyolysis, and abnormality of chromosomes.

Some of the 17 cobalt complex salts also showed karyorrhexis, karyolysis, cytolysis, and pyknosis 10 to 24 hours after administration, associated with an inflammatory response.  $\text{CO}(\text{NH}_3)_4\text{Cl}_2\text{Cl}$  was active (33).

The response to chromomycin  $\text{A}_3$  is characterized by late destruction of tumor cells, accompanied by an infiltrate of neutrophils and monocytes (7, 8). When 5  $\mu\text{g}$  per kg was injected no changes occurred, but when 7.5  $\mu\text{g}$  per kg was given there was moderate destruction of tumor cells after injection. At 10 to 20  $\mu\text{g}$  per kg, destructive changes occurred 24 hours later, with a marked emigration of reactive cells. However, the tumor cells remained almost unchanged for 6 hours even at the dose of 10 to 20  $\mu\text{g}$  per kg. This delayed effect is characteristic of the effectiveness of the drug, and the other is the sharp dose response at the 5 to 10  $\mu\text{g}$  per kg level. A few Nitromin-like effects were also observed, and multinucleated giant cells appeared.

The mechanism of biochemical action of chromomycin  $\text{A}_3$  suggests a specific inhibition of RNA, probably as an inhibitor of nucleolar RNA synthesis (34). After incubating the solution of chromomycin  $\text{A}_3$  with tumor or normal cell suspensions, the antibiotic was isolated from the supernatant and precipitated cells. Chromomycin  $\text{A}_3$  was identified chemically, and quantitative and qualitative determinations were made, using paper chromatography. There appears to be some selectivity for chromomycin  $\text{A}_3$  between tumor cells and normal cells in *in vitro* experiments. Chromomycin  $\text{A}_3$  has an affinity for lymph nodes, but the host-damaging activity, expressed by the influence upon the formation of nonspecific antibodies and the effect on the reticuloendothelial system

(RES), is minimum at the dose usually given. Another frequent change is vacuolization of cytoplasm. The effects on cytoplasm should be further studied. PAS-staining has been reported (35), and methyl green-pyronin staining and other cytochemical methods have been tested as routine procedures.

The molecular weight of chromomycin A<sub>3</sub> is relatively high. The C, H, and O determinations and results of chemical research were presented at the International Union of Pure and Applied Chemistry meeting at Kyoto, in April, 1964.

As we stated previously, the number of "false positives" in cytological screening may be relatively few, if the worker is well trained in pathology and knows the limitation of Giemsa-stained specimens, but "false negatives" require further study. With an antimetabolite such as 6-mercaptopurine it is difficult to show the characteristic effects with this method, although the effects of aminopterin (36) indicate that after 48 hours there is pyknosis, karyorrhexis, and especially vacuolar degeneration of cytoplasm and the changes continue for 3 to 4 days. Attempts to express the cytological effects caused by other typical antimetabolites and to establish their characteristic changes have so far been unsuccessful.

#### APPROACHES TO THE CRITERIA OF "CURE" OR PROLONGATION OF SURVIVAL TIME OF YOSHIDA SARCOMA-BEARING RATS

Our criteria are based on the cytological changes in tumor cells and the prolongation of survival time of the tumor-bearing animal. Therefore, it is essential that the tumor and the animals used for the screening experiments are compatible. For instance, more than 20 percent "no takes" or regression in a group of control animals should be considered presumptive evidence of incompatibility. If possible, the animals used should be homogeneous and we attempted to establish a rat colony highly susceptible to the Yoshida sarcoma.

##### Establishment of "Donryu Rats" (37)

In 1951, Dr. R. Sato started breeding a pair of rats obtained from a dealer at the Saitama Prefecture and succeeded in developing a rat colony highly susceptible to the Yoshida sarcoma. T. Yoshida, Satoh, and Kaziwara then began to breed a few rats and obtained 100 percent lethal takes of the tumor. They then started an extensive breeding of the colony and got analogous rats for the Yoshida sarcoma; in 1958 they were designated "Donryu rats" (table 12).

##### Mass Production and Brother $\times$ Sister Mating of Donryu Rats

Table 13 describes the availability of the rat colony for the transplantation of the tumor as it has been mass-produced for screening experiments since 1951, with sib-mating continued from 1953 to date <sup>3</sup> (38).

<sup>3</sup> At the animal breeding center attached to the Hikari Factory, Takeda Chemical Industries, Ltd., with the designation of Donryu-rats/H under the direction of Dr. Kaneko and Mr. Asakura.

TABLE 12.—Summary of transplantation and survival time of Donryu rats intraperitoneally transplanted with Yoshida sarcoma

Strain	Number of rats injected	Percent of takes (transplantation rate)	Survival time (days)
Donryu, R. Sato Farm	927	99.9	About 8
Donryu, Osawa Farm	1036	100.0	Mode 7
Donryu, R. Sato Farm	122	100.0	Average 8.1
Donryu, Osawa Farm	164	99.4	Average 6.5
Donryu, Osawa Farm	150	100.0	About 8
Donryu, Hikari Factory	686	99.1	About 8
Japanese random colony	1724	88.3	Average 11

TABLE 13.—Breeding of Donryu rats (January, 1958)

Name of facility	R. Sato Farm	Osawa Farm	Hakari Factory, Takeda Chemical Industries, Ltd.
Start of breeding	1950	1950	1953
Breeding system	Random	Random	Random and brother × sister (F-11)
Numbers supplied per month	50	250	600
Supply to	None	Jatrochemical Institute Sasaki Institute Fukushima University Gerontology Institute	Takeda Institute
Possibility of increase in supply.	Yes	Yes, up to 500/month	Yes, up to 1300/month
Easiness of breeding	Yes	No	Yes

The susceptibility of the offspring was successively checked before the second litter was put into the nucleus colony for random breeding, which consisted of 6 groups with uniform genetic variability. Although genetic examinations may deserve further observation, Donryu rats are so satisfactory in their uniform response to the Yoshida sarcoma that they have always been used for screening experiments in our laboratory.

#### Survival Standard of the Yoshida Sarcoma in Donryu Rats and Expression in Percent Survival

Transplantability and survival have been carefully examined and summarized (tables 14 and 15).

Kaziwara (39) first described the usefulness of "percentage survival" to express the transplantation rate and survival time by the same single figures, after noting Hauschka's paper (40) on the death curve of mice with a parasitic infection. This method has been used extensively in Japan, and it should be stressed that the number of animals used is always recorded so as to get correct information from the figures. If the curve reaches the bottom it means 100 percent "lethal takes," and if the curve runs parallel, horizontally, the transplantability rate is not 100 percent. In other words, the percentage survivals can express the rate of "lethal takes" and the survival time in days in one figure (41).

TABLE 14.—Percent survival of 1,062 Donryu rats intraperitoneally transplanted with the Yoshida sarcoma, 1953–1957 [Hiroshi Satoh, Jatrochemical Institute, Tokyo]

Year	1953	1954	1955	1956	1957	Total
Number of animals	19	302	305	220	216	1,062
Effective No. of animals	18	297	293	216	212	1,036
Survival, days	4		1(99.7)			1(99.9)
	5	13(95.6)	9(96.6)	3(98.6)		25(97.5)
	6	41(81.8)	58(76.8)	18(90.3)	17(92.0)	134(84.6)
	7	7*(61.1)†	79(55.2)	56(64.4)	63(62.3)	297(55.9)
	8	8(16.6)	73(30.6)	70(21.6)	53(39.8)	261(30.7)
	9	2(5.6)	55(12.1)	35(9.6)	47(18.1)	174(13.9)
	10	1(0)	26(3.4)	20(2.7)	26(6.0)	92(5.0)
	11		5(1.7)	7(0.3)	9(1.9)	28(2.3)
	12		5(0)	1(0)	7(6.6)	12(1.2)
	13			2(0.9)	4(4.7)	1(1.1)
	14				1(4.2)	1(1.1)
	15			1(0.5)	3(2.8)	4(0.7)
	16					
	17				1(2.4)	1(0.6)
	18				2(1.4)	2(0.4)
	19				1(0.9)	1(3.3)
	20			1(0)		1(0.2)
	34				1(0.5)	1(0.1)
	36				1(0)	1(0)
Transplantation rate	100	100	100	100	100	100

\* Number of rats that died.

† Figures in parentheses indicate percent survival.

TABLE 15.—Changes in survival time of Donryu rats with various numbers of inocula [Hiroshi Satoh, Jatrochemical Institute, Tokyo]

Cells in inoculum	10 <sup>2</sup>	10 <sup>3</sup>	5 × 10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>7</sup>
Survival, days							
	5						
	6						4(60)
	7					2(80)	4(20)
	8					4(40)	2(0)
	9					4(0)	
	10			1(90)	6(40)		
	11			3(60)	4(0)		
	12			3(30)			
	13	1*(90)†	2(80)	2(10)			
	14		1(90)	1(70)			
	15	2(70)	1(80)	3(40)			
	16	2(50)	3(50)	2(20)			
	17	2(30)	3(50)	2(0)			
	18		1(10)				
	19	2(10)					
	20						
	21		1(0)				
Transplantation rate	90	100	100	100	100	100	100

\* Number of rats that died.

† Figures in parentheses indicate percent survival

## Spontaneous Regression of Yoshida Sarcoma

During the serial transplantation experiments with the noninbred Donryu rats, there has been a low percentage of spontaneous regressions of the tumor. These infrequent regressions, not attributable to any treatment, might possibly have been caused by many factors, such as age, sex, nutritional conditions, hormonal imbalance, infections of host animals, or changes in the tumor cell itself. However, at present, the genetic heterogeneity between the rats and Yoshida sarcoma is considered the most probable factor causing spontaneous regressions. Although the Donryu rats were selected because of their high susceptibility toward the tumor, the tumor was derived from an impure rat presumably different from the ancestor of the Donryu rats. To test whether the regression correlates with the hereditary factors of the host and the tumor, transplantation experiments were undertaken with the offspring of tumor-negative parents (42). The results obtained for 5 generations are summarized in table 16.

TABLE 16.—Results of transplantation of the Yoshida sarcoma in the offspring of tumor-negative Donryu rats

Generation	Number of offspring	Rats dying with tumor	Incomplete regression*	Complete regression†
F <sub>1</sub>	9	2 (22.3)‡	6 (66.6)‡	1 (11.1)‡
F <sub>2</sub>	21	17 (81.0)	1 (4.7)	3 (14.3)
F <sub>3</sub>	21	5 (23.8)	15 (71.5)	1 (4.7)
F <sub>4</sub>	18	6 (33.3)	10 (55.5)	2 (11.2)
F <sub>5</sub>	6	1 (16.6)	4 (66.7)	2 (33.7)
Total	75	32 (42.6)	35 (46.7)	9 (10.7)

\*Tumor cells once proliferated for about 7 days and regressed later.

†Tumor cells disappeared within a few days.

‡Figures in parentheses indicate percent.

These data suggest that the tumor regression in the Donryu rats might be caused by an immune response, resulting from the antigenic differences between tumor and host. In this case, it is probable that histocompatibility-like factors might possibly be associated with their antigenic dissimilarities.

Recently some authors reported the presence of a histocompatibility hemagglutinin in several inbred strains of rats and their derived tumors, and stressed its role in the homotransplantation of tumors (43, 44). This type of immune response may correspond to that described by Medawar (45) in homotransplantation immunity of skin grafting. Similar phenomena were demonstrated by Matsumoto, using Yoshida sarcoma and Wistar rats (46). However, further explanation on this point is impossible due to the lack of genetic background of the Yoshida sarcoma. It is apparent from the present data that the genetic constitutions are associated with the spontaneous regression of the tumor.

Infection With *Trypanosoma lewisi*

Another important factor in the tumor regression is spontaneous infection in the host by protozoa (40, 47, 48). Rats with spontaneously regressed tumors have often been infected with *Trypanosoma lewisi*. This infection can be eliminated by short-term passage of the contaminated tumor ascites in mice. It thus is probably not an important factor in tumor regression. The possible relationship between this infection and transplantation of Yoshida sarcoma in rats is shown in table 17.

TABLE 17.—Transplantability and survival time of Yoshida sarcoma-bearing rats with or without infection with *Trypanosoma lewisi*

	Number of rats	Number of takes	Percent of takes	Average lifespan (days)
Inoculation of tumor alone	51	51	100	11.33
Inoculation of tumor in combination with infection of <i>T. lewisi</i>	20	18	90	11.05
Inoculation of tumor after infection of <i>T. lewisi</i>	30	19	63.3	10.59

Spontaneous regressions of tumor may also be caused by accidental infections with some viruses (49) and fungi (50). In these cases, specific or nonspecific host defense reactions, including RES response and hormonal imbalance, might play a very important role (51).

Variability of the tumor itself during the course of serial transplantation is another possible factor for tumor regression. Changes in the phenotypic character of the Yoshida sarcoma have been described by some investigators (46, 52). However, it is not within the scope of the present paper to discuss the spontaneous regression phenomenon on the basis of the changes in tumor cells. It is not unreasonable to postulate that changes in genetic constitutions of tumor cells during serial transplantation might possibly contribute to the inadequacy of host conditions for the tumor growth.

## Possible Appraisal Between Spontaneous Regressions and Chemotherapy Screening

The phenomenon of spontaneous regression mentioned might interfere with the practical evaluation of the "cure" of animals in routine chemotherapy screenings. Specific criteria are therefore necessary for evaluating survival, and care must be taken to minimize all the possibilities of spontaneous regressions in the tumor-host systems used.

The Yoshida sarcoma-Donryu rat system employed in the present experiment seems to be satisfactory for screening chemotherapeutic compounds. In experimental chemotherapy studies, one must consider whether the "cure" of animals is actually due to the disappearance of tumor cells, because of treatment with the chemotherapeutic agent, or whether there was spontaneous regression of the tumor.

### Some Immunological Aspects of "Cure" Induced by Treatment With Chemotherapeutic Agents and the Problem of Host Conditioning

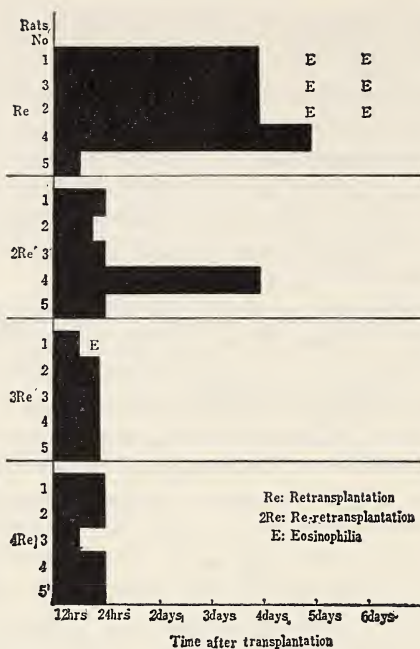
The screening experiments have shown that if the animals with Yoshida sarcoma were cured by chemotherapy, they would thereafter acquire resistance to the reimplantation of the same tumor. This phenomenon may be understood as a reflection of the host immune reaction elicited as a result of the destruction of the tumor cells by chemotherapeutic drugs. It seemed therefore to be of interest to study the nature of "cure" induced by the treatment with some antitumor agents.

Because of its effectiveness on the Yoshida sarcoma, Nitromin was used in the present study. When Nitromin was inoculated into the tumor-bearing rats on the 3d day after tumor transplantation, the animals were cured of the tumor, and there was marked destruction of tumor cells. The animals thus cured were challenged with the same tumor several times, and the growth or disappearance of the implanted tumor cells was successively followed *in situ* (53). From this study, it became evident that the animals once cured of the tumor by treatment with Nitromin were highly resistant to following challenges of the same tumor (text-figs. 1 and 2). The more often the challenges were repeated, the more rapidly the tumor cells disappeared. After the first retransplantation (challenge), the tumor cells survived for about 5 days in the peritoneal cavity of a resistant host, and in the second or third challenges they disappeared within 24 hours after implantation. These phenomena resemble "first- and second-set reactions" in tissue transplantation immunity.

Cytological changes, observed during the course of disappearance of tumor cells in the Nitromin-cured rats, were followed successively by use of phase-contrast microscopy. Early changes in tumor cells were: condensation of nucleoli, granular appearances in the cytoplasm, and distinct irregularity of nuclear membrane subsequently followed by fragmentation and pyknosis of nuclei. Thereafter, tumor cells became small, globular-shaped, and finally changed into cell debris. Accompanying these changes were striking host cellular responses. The predominant host cells appearing in the ascites were neutrophils, macrophages, and monocytes. It was also observed that the reactive cells apparently adhered to tumor cells (figs. 1 and 2) and seemed to be a factor in the destruction of the tumor cells. After or just before disappearance of the tumor cells, an increased number of eosinophils has often been observed in the ascites (text-fig. 2). These host responses may be an inflammatory reaction due to the immunological responses.

Studies on the cytotoxic action of sera, taken from the "cured" rats, on Yoshida sarcoma cells have been studied *in vitro* and *in vivo*. It has been shown that sera from rats after repeated challenges might be somehow cytotoxic upon the tumor cells (table 18).

These results suggest that both cellular and humoral factors may be important in the disappearance of the tumor cells in the "cured" rats. Therefore, it is reasonable to assume that some chemotherapeutic agents may not only attack the tumor cells directly but also stimulate some

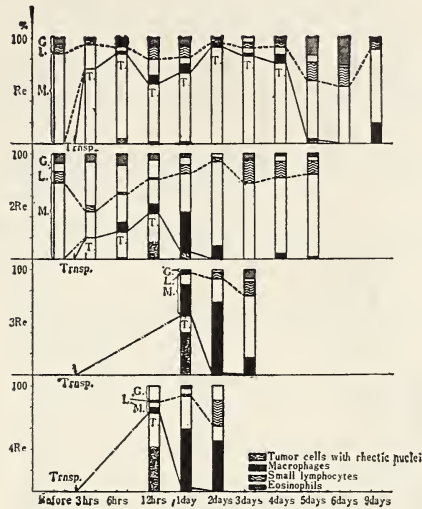


TEXT-FIGURE 1.—Survival of tumor cells in ascites of Yoshida sarcoma rats treated with Nitromin.

factors which facilitate the host in such a way that the tumor-host relationship is altered in favor of the host. Although further experimental evidence is needed, the following speculation may be possible: Ordinarily, tumor cells may be recognized as "self" in the susceptible hosts, but when the tumor cells are treated by chemotherapy, the antigenicity of the tumor cells may be altered to "not-self," thus leading the host animals to an immune state against the next challenge of the same tumor. There seems to be an analogy between acquired immunity of "Nitromin-cured" rats and the immune reaction of guinea pigs, which was evoked by the injection of a denatured autologous  $\gamma$ -globulin (54).

Although the true nature of these phenomena is not fully clarified, it should be emphasized that "host factors" are very important in experimental cancer chemotherapy.

The screening methods employed in our laboratory during the past decade have provided many substances for cancer chemotherapy. However, many are decidedly harmful both to tumor and normal tissues. Therefore, these compounds are not considered to be true anticancer chemotherapeutic agents. From the practical point of view, it is important that a choice be made of compounds which are selective on tumor cells, but not on normal tissues. In this respect, host defense factors may be most important.



TEXT-FIGURE 2.—Changes in cellular composition of ascites of Nitromin-cured Yoshida sarcoma rats after retransplantation of  $2 \times 10^5$  cells. G = granulocytes; L = small lymphocytes; M = monocytes and macrophages; and T = tumor cells.

Recently many attempts have been made to stimulate or to enhance general host defense mechanisms which are also inhibitory against tumor growth: Zymosan (55), BCG (56), bacterial lipopolysaccharides (57), and methylcellulose (58). It was reported that pretreatment with various tissues including normal and malignant (59) or extracts of these tissues (60) were effective in inhibiting tumor growth. Studies along these lines are now in progress in our laboratory.

The present paper has described several compounds tested and screened primarily for their activity in causing unusual cytological changes in Yoshida sarcoma, based on our experiences during the past 10 years. The cytological changes were classified practically and conventionally for the primary screening of several unknown, new compounds into several categories, such as alkylating-agent effect, colchicine effect, and others. Several compounds with an activity that caused cytological changes, similar to that of known reference compounds, were obtained and further karyological, biochemical activities, and submicroscopic changes caused by such compounds are now being extensively studied.

Available data in the field of immunology suggest that nonspecific or specific immune responses elicited by various means might possibly participate in a host defense against cancer. Further prospects for cancer chemotherapy will be centered on improved methods for screening adequate agents that may enable the host to resist the cancer growth.

In conclusion, the following words are, as presented by T. Yoshida, cited as the final aim for cancer chemotherapy, "I hope indeed that a way would be found to depress, not necessarily to kill, its impudent growth, in order that man may live happily in a state of co-existence."

TABLE 18.—Neutralization of Yoshida sarcoma by sera from cured rats

Serum from	Survival days of rats inoculated* with tumor cells:									
	$1.7 \times 10^5$		$1.7 \times 10^4$		$1.7 \times 10^3$		$1.7 \times 10^2$		$1.7 \times 10$	
Nitroin-cured Donryu	11	13	20	14	14	15	—	—	—	—
Spontaneously regressed Donryu	14	15	43	—	—	—	—	—	—	—
Normal Donryu	8	9	10	9	10	13	12	12	—	—
Normal Sprague-Dawley	9	14	11	15	—	—	11	14	14	24
	11	13	17	13	13	—	13	14	20	—
	9	10	16	10	16	—	12	13	16	—
Rabbits	17	—	—	18	—	—	—	—	—	—
Control, phosphate-buffered saline †	9	9	17	11	—	—	11	17	—	—

\* 0.1 ml of each cell suspension was mixed with 0.5 ml of sera; after incubation for 1 hour at 37° C, 0.1 ml of the mixtures was injected intraperitoneally into Donryu rats.

† Dilution medium pH 7.4.

## CONCLUDING REMARKS

In deciding if a cytological method of a given transplantable tumor is worthwhile as a tool for cancer chemotherapy screening we must consider that:

(a) The morphological characteristics of the tumor must be known and the variations in the control established.

(b) The cytological responses of the tumor to the known cancerostatic agents must be checked and the rate of "false positives" and "false negatives" determined.

(c) The results of clinical application of the compounds must be evaluated by using the system given.

Point (a) has been discussed elsewhere, and point (c) will be referred to in various clinical papers on Nitromin, carzinophilin, mitomycin, and chromomycin A<sub>3</sub> (Toyomycin). Point (b) was discussed in the present paper in which we found "cure" or prolongation of the lifespan of laboratory animals. Almost all morphologically positive compounds may also be positive in checking the lifespan, but several reference compounds, such as antimetabolites, are difficult to evaluate through this particular tumor system. We should, of course, know the limitations of the tumor system.

## REFERENCES

- (1) YOSHIDA, T.: The Yoshida Sarcoma, an ascites tumor. *Gann* 40: 1-20, 1949.
- (2) YOSHIDA, T., SATO, H., NAGASAWA, B., ATSUMI, A., SATO, H., KOBAYASHI, K., HOSOKAWA, K., and NAKAMURA, K.: Experimental studies on chemotherapy of malignant growths using Yoshida Sarcoma (1). *Gann* 41: 93-95, 1950.
- (3) ISHIDATE, M., KOBAYASHI, K., SAKURAI, Y., SATO, H., and YOSHIDA, T.: Experimental studies on chemotherapy of malignant growth employing Yoshida Sarcoma animals. II. The effect of N-oxide derivatives of nitrogen mustard. *Proc Jap Acad* 27: 493-503, 1951.
- (4) TATSUOKA, S., *et al.*: Chemical and biological studies upon Sarcomycin and related compounds. *Gann* 47: 364-366, 1956.
- (5) KOGA, F.: Experimental treatment of malignant tumor with antibiotics. II. Experimental treatment with a new antibiotic, Carcinophilin. *J Antibiot Ser B* 7 (8): 275-282, 1954.
- (6) USUBUCHI, I., OHOSHI, S., TSUCHIDA, R., and TANABE, H.: The effect of mitomycin C on the growth of a variety of rat and mouse tumors. *Gann* 49: 209-222, 1958.
- (7) KAZIWARA, K., WATANABE, J., KOMEDA, T., and USUI, T.: Further observations on the inhibiting effects of chromomycin A<sub>3</sub> on transplantable tumors. *Cancer Chemother Rep* 13: 99-106, 1961.
- (8) USUI, T., and KAZIWARA, K.: Cytological analysis of the effect of a cytotoxic antibiotic chromomycin A<sub>3</sub> on Yoshida ascites sarcoma cells. *Ann Rep Takeda Res Lab* 20: 165-205, 1961.
- (9) KOPROWSKA, I.: Personal communication (1953).
- (10) Protocols for screening chemical agents and natural products against animal tumors and other biological systems. *Cancer Chemother Rep* 25: 1-66, 1962.

- (11) ISHIDATE, M., SAKURAI, Y., YOSHIDA, T., and SATO, H.: Experimental studies on chemotherapy of malignant growth employing Yoshida Sarcoma animals (V). *Gann* 45: 484-468, 1954.
- (12) ISHIDATE, M., SAKURAI, Y., OWARI, S., YOSHIDA, T., SATOH, H., and IMAMURA, H.: Experimental studies on chemotherapy of malignant growth employing Yoshida Sarcoma animals. XII. Carcinostatic action of sulfonic ester. *Gann* 47: 372-375, 1956.
- (13) DRUCKREY, H., SCHMÄHL, D., DANNEBERG, P., KAISU, K., NIEPER, H.A., LO, H. W., and MECKE, R.: Vergleichende Prüfung der Chemotherapeutischen Wirkung von N-oxyl-Lost und anderen alkylierenden Substanzen auf Tumoren von Ratten. *Arzneimittelforschung* 6: 539-550, 1956.
- (14) NISHIZUKA, Y.: The effect of hormone and anticancer substances upon the lymphsarcoma cells of mice (in Japanese). "Chemotherapy of Cancer" (Takeda, K., ed.). Ishiyaku, Tokyo, 1957, pp 217-228.
- (15) USUBUCHI, I., OBOSHI, S., TSUCHIDA, R., and TANABE, H.: Further studies on the effect of Mytomyacin C upon experimental tumors. *Gann* 49: Suppl 14, 1958.
- (16) WHEELER, G. P.: Studies related to the mechanisms of action of cytotoxic alkylating agents. A review. *Cancer Res* 22: 651-688, 1962.
- (17) EIGUSTI, O. J., and DUSTIN, P.: Colchicine, in *Agriculture, Medicine, Biology and Chemistry*. Ames, Iowa State College Press, 1955.
- (18) SATO, H.: Cited in "Yoshida Sarcoma." (Yoshida, T., ed.). Neiraku, Tokyo, 1952, p 171.
- (19) ISHIDATE, M., WATANABE, J., ARAMAKI, Y., and KAZIWARA, K.: Studies on effects of Colchicine derivatives upon Yoshida Sarcoma cells. *Ann Rep Takeda Res Lab* 16: 90-99, 1957.
- (20) JOHNSON, I. S., WRIGHT, H. F., SVOBODA, G. H., and VLANTIS, J.: Antitumor principles derived from *Vinca rosea* Linn. I. Vincal leukoblastine and Leurosine. *Cancer Res* 20: 1016-1022, 1960.
- (21) CUTTS, J. H.: The effect of Vincal leukoblastine on dividing cells in vivo. *Cancer Res* 21: 168-172, 1961.
- (22) USUI, T., and KAZIWARA, K.: Colchicine-like effect of several new compounds upon the Yoshida Sarcoma cells. II. Effect of phenylazopyrimidine and alkaloids of *Vinca rosea*. *Proc Jap Cancer Ass*, p 141, 1962.
- (23) TANAKA, K., SUGUWA, T., KUWADA, U., IMAI, K., MORINAGA, M., WATANABE, J., KOMEDA, T., USUI, T., YOKOTANI, H., ITÔ, H., HEMMI, S., KATO, M., MIMA, H., and KAZIWARA, K.: Studies on nucleic acid antagonist V. I. Relationships between chemical structure and antitumor activity in 5-phenylazopyrimidine. *Ann Rep Takeda Res Lab* 22: 192-216, 1963.
- (24) USUI, T., and KAZIWARA, K.: Cytological studies of the effects of Py-122 glutamate on the dividing cells of the Yoshida Sarcoma and those of the ascites hepatoma. *Ann Rep Takeda Res Lab* 22: 217-221, 1963.
- (25) REGELSON, W., and HOLLAND, J. F.: Initial chemical study of parenteral methylglyoxal bis (guanyldihydrazone) diacetate. *Cancer Chemother Rep* 11: 81-86, 1961.
- (26) HIBINO, S.: Chemotherapy of acute leukemia in Japan. *Cancer Chemother Rep* 13: 141-144, 1961.
- (27) KAZIWARA, K., USUI, T., WATANABE, J., and KOMEDA, T.: Metaphase arrest-effect of several compounds upon Yoshida sarcoma. III. Effect of xenamine and its derivatives. *Proc Jap Cancer Ass*, 1963, p 129.
- (28) CAVALLINI, G.: Chimiothérapie antivirale. *Med Hyg, Geneva* 514: 640-641, 1961.
- (29) *Neue Arzneimittel und Spezialitäten*. 10 (11): 154, 1963.
- (30) CARDINALI, G., and ENEIN, M. A.: Studies on the antimitotic activity of Leurocristine (Vincristine). *Blood* 21: 102-110, 1963.

- (31) YOSHIDA, T., SATO, H., NAGASAWA, B., ATSUMI, A., KOBAYASHI, K., HOSOKAWA, K., NAKAMURA, K., ISHIDATE, M., and SAKURAI, Y.: Experimental studies on chemotherapy of malignant growths using Yoshida sarcoma (I). *Gann* 41: 93-96, 1950.
- (32) ISAKA, H.: The effect of emetine hydrochloride upon the Yoshida Sarcoma. *Gann* 41: 165-168, 1950.
- (33) KAZIWARA, K.: The effects of cobaltic complex salts upon the Yoshida sarcoma. *Gann* 41: 168-170, 1950.
- (34) YANO, M., KUSAKARI, T., and MIURA, Y.: Intracellular transfer of nucleic acids. III. Fate of nucleic acids in rat ascites hepatoma cells. *J Biochem* 53 (6): 461-464, 1963.
- (35) SATO, H., NOUCHI, F., YOSHIDA, T., and ISHIDATE, M.: Experimental studies on chemotherapy of malignant growth employing Yoshida sarcoma animals. XVI. Determination of minimum effective dose (MED) of Nitromin and other compounds by means of cytochemical detection of the PAS positive substances. *Gann* 48: 403-404, 1957.
- (36) BURCHENAL, J. H.: Current status of clinical chemotherapy. I. Antimetabolites. *Current Res in Cancer Chemother Rep* 4: 3-30, 1956.
- (37) YOSHIDA, T.: Donryu-rat, a colony of Japanese noninbred rat which is highly susceptible to the intraperitoneal transplantation of the Yoshida sarcoma. *Bull Exp Animals* 7: 85-91, 1958.
- (38) KAZIWARA, K., WATANABE, J., KOMEDA, T., KAWAJI, K., MORITA, T., KIMURA, R., ASAKURA, H., KODAMA, A., and OKIMURA, H.: On the Yoshida sarcoma susceptibility of randombred and inbred Donryu rats. *Ann Rep Takeda Lab* 21: 139-156, 1962.
- (39) KAZIWARA, K., ISAKA, H., NAKAMURA, K., ARUJI, T., KAISE, A., ODASHIDA, S., and SATOH, H.: Studies on the ascites hepatoma. II. On the transplantation rate of the ascites hepatoma intraperitoneally transplanted. *Gann* 44: 307-309, 1953.
- (40) HAUSCHKA, T. S.: *Trypanosoma cruzi* in the treatment of mouse tumors. *Cancer Res* 7: 717, 1947.
- (41) KAZIWARA, K., KOMEDA, T., USUI, T., WATANABE, J., MORITA, T., and KIMURA, T.: Studies on the screening method of tumor chemotherapy. II. Transplantation rate and survival days of seven rat ascites tumors and five mouse ascites tumors. *Ann Rep Takeda Lab* 20: 72-113, 1961.
- (42) KAZIWARA, K.: Complete and incomplete regression of the Yoshida sarcoma and their relations to hereditary constitution of rats. *Gann* 43: 242-245, 1952.
- (43) BOGDEN, A. E., and APTEKMAN, P. M.: The R-1 factor, a histocompatibility antigen in the rat. *Cancer Res* 20: 1372-1382, 1960.
- (44) MATSUMOTO, T.: Antigenicity of a methylcholanthrene-induced spindle sarcoma of inbred rats. *Jap J Cancer Clin* 8: 577-583, 1962.
- (45) MEDAWAR, P. B.: Reactions to homologous tissue antigens in relation to hypersensitivity. In "Cellular and Humoral Aspects of the Hypersensitive States" (Lawrence, H. S., ed.). New York, Hoeber-Harper, 1961, pp 504-529.
- (46) MATSUMOTO, T.: Immunological studies of tumors, V. Further studies of antigenic differences between two sublines of the Yoshida sarcoma. *Gann* 52: 57-65, 1961.
- (47) HAUSCHKA, T. S., SAXE, L. H., and BLAIR, M.: *Trypanosoma cruzi* in treatment of mouse tumors. *J Nat Cancer Inst* 7: 189-197, 1947.
- (48) JEDELOO, G. C., LIGNAC, G. O. E., LIGTENBERG, A. J., and VAN THIEL, P. H.: The biotherapeutic action of *trypanosoma cruzi* on tar cancer of mice. *J Nat Cancer Inst* 10: 809-813, 1950.
- (49) SOUTHAM, C. N.: Present status of oncolytic virus studies. *Trans NY Acad Sci* 22: 657-673, 1960.
- (50) MANKOWSKI, Z. T., DILLER, I. C., and FISHER, M. E.: The effect of various fungi on mouse tumors with special reference to sarcoma 37. *Cancer Res* 17: 382-386, 1957.

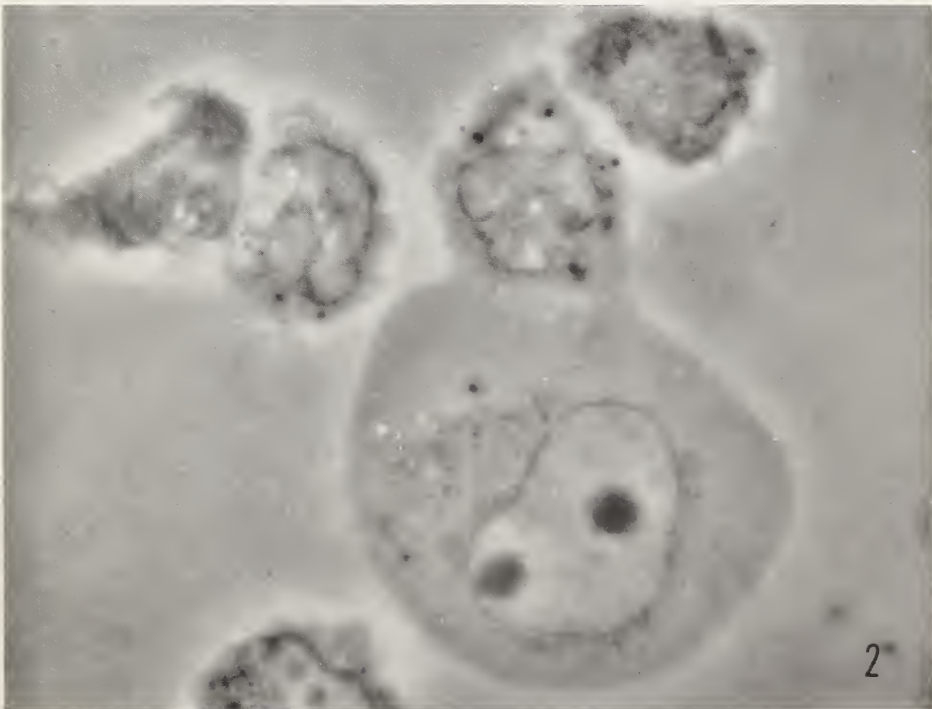
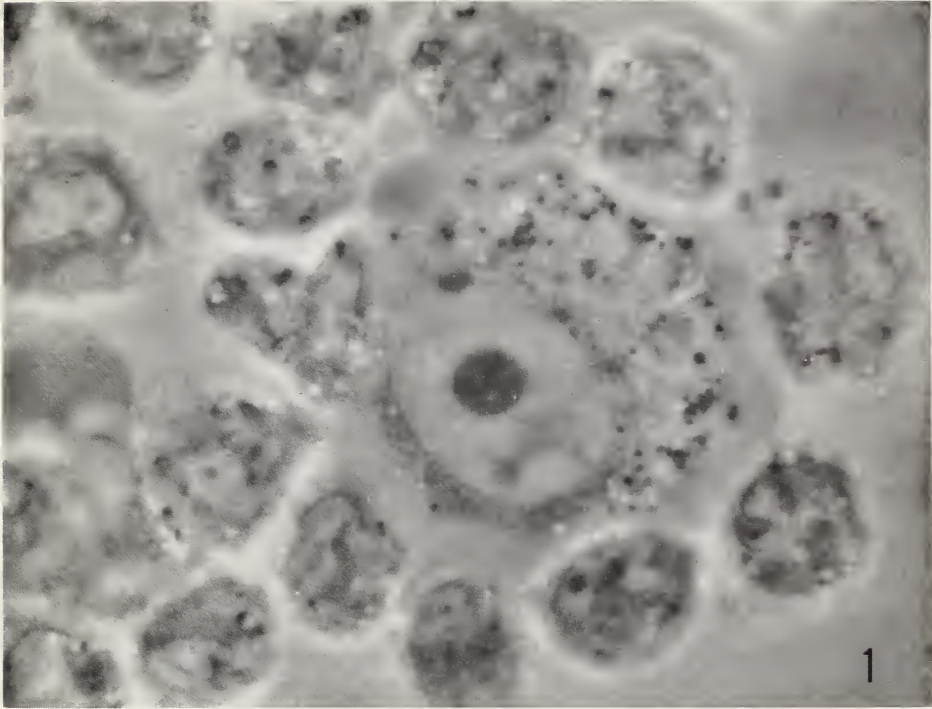
- (51) FURTH, J.: Influence of host factors on the growth of neoplastic cells. *Cancer Res* 23: 21-34, 1963.
- (52) SASAKI, M.: Cytological studies of tumors. XXVIII. Some experimental studies in two sublines of the Yoshida sarcoma, with special consideration of the deviation of sublines. *Cytologia* 25: 69-85, 1960.
- (53) KAWAJI, H., OTSU, K., and KAZIWARA, K.: Changes observed during Yoshida sarcoma rejection in Nitromin cured Yoshida sarcoma rats and their splenomegaly. *Ann Rep Takeda Res Lab* 22: 254-287, 1963.
- (54) ROBERT, T. M., MILLER, F., and BENACERRAF, B.: Sensitization to denatured autologous gamma globulin. *J Exp Med* 115: 253-273, 1962.
- (55) BRADNER, W. T., CLARKE, D. A., and STOCK, C. C.: Stimulation of host defense against experimental cancer. I. Zymosan and S-180 in mice. *Cancer Res* 18: 347-351, 1958.
- (56) OLD, L. J., CLARKE, D. A., and BENACERRAF, B.: Effect of *Bacillus Calmette-Guérin* infection on transplanted tumors in mouse. *Nature (London)* 184: 291-292, 1959.
- (57) MALKIEL, S., and HARGIS, B. J.: Influence of *Bordetella pertussis* on host survival following S-180 implantation. *Cancer Res* 21: 1461-1464, 1961.
- (58) LAZAR, A., and LAZAR, D. C.: Effect of methylcellulose on the Murphy-Sturm lymphosarcoma in rats. *J Nat Cancer Inst* 28: 1255-1267, 1962.
- (59) SATO, H.: Personal communication (1962).
- (60) SZENT-GYORGYI, A., HEGYELI, A., and McLAUGHLIN, J. A.: Constituents of the thymus gland and their relation to growth, fertility, muscle, and cancer. *Proc Nat Acad Sci USA* 48: 1439-1442, 1962.

PLATE

## PLATE 50

FIGURE 1.—Mononuclear cells adhere around the tumor cell. *Note* the condensation of nucleoli, distinct margination of nucleus, and granulation in the cytoplasm. Phase-contrast micrography.  $\times 1,550$

FIGURE 2.—A neutrophil adheres to the degenerating tumor cell showing distinct margination of nucleus, condensation of nucleoli, and coagulation of cytoplasmic granules. Phase-contrast micrography.  $\times 1,800$











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